ENHANCED DROUGHT TOLERANCE THROUGH PLANT GROWTH PROMOTING RHIZOBACTERIA AND MICROBIOME ENGINEERING APPLICATIONS

A Dissertation

by

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ABSTRACT

Water is a major limiting resource in agriculture worldwide, restricting crop yields in approximately 70 percent of arable farmlands. My goal was to alleviate drought stress in grasses using plant growth-promoting rhizobacteria (PGPR) and host-mediated microbiome engineering (HMME) of the rhizosphere. In the summers of 2016 and 2017, we collected bermudagrass rhizospheres from El Paso, TX for PGPR bioprospecting. Two novel isolates, *Bacillus* sp. (12D6) and *Enterobacter* sp. (16i) , were shown to delay the onset of drought stress in wheat (*Triticum aestivum* subsp. *aestivum* cultivar TAM 111) and maize (*Zea maize* cultivar B73) seedlings. Roots inoculated with these PGPR resulted in statistically significant alterations in root system architecture traits associated with drought tolerance in a host-specific manner. In the second part of this study, I employed host-mediated microbiome engineering to confer a generational increase in drought tolerance of wheat seedlings. In this host-centric artificial selection process, the wheat rhizosphere was sub-selected based on host phenotypic tolerance after a prolonged water deficit. After six rounds of microbiome engineering, seedlings growing in the engineered microbiome withstood an additional 5 days of water deficit compared to the initial microbiome. The engineered microbiome demonstrated statistically significant alterations in root system architecture and increases in water retention. Next generation sequencing of rounds 0, 3, and 6 using the 16S rRNA gene followed by bioinformatic analyses revealed taxonomic increases in Proteobacteria at the phylum level and Betaproteobacteria at the class level, progressively decreasing α – diversity of bacterial community, and changes in the functional metagenome for cell motility, signaling, and metabolism. Overall, findings from both studies improve the understanding of the ecological and evolutionary implications of plant-microbe interactions in a water deficient environment, with potential applications that can be directly used for mitigating drought stress in cereal crops.

DEDICATION

I dedicate this dissertation to my wife, Calli Jochum, who provided me with encouragement and support during graduate school. To my child, Michael Donovan Jochum III, may this dissertation show you that with hard work and dedication, anything is possible. Finally, I dedicate this dissertation to all the professors that mentored, supported, and believed in me.

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The WinRHIZO data analyzed for Chapter 2 and 3 was acquired using the flatbed scanner and software provided by Professor Elizabeth Pierson. The statistical analyses depicted in Chapter 2 and 3 were conducted with guidance from Dr. Young-Ki Jo of the Department of Plant Pathology and Microbiology and were published in 2018 in the articles listed in the Biographical Sketch.

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NOMENCLATURE

ACC	1-Aminocyclopropane-1-Carboxylate
ANOVA	Analysis of Variance
BLAST	Basic Local Alignment Search Tool
CEC	Cation Exchange Capacity
CFU	Colony Forming Unit
CTAB	Cetyl Trimethyl Ammonium Bromide
DADA2	Divisive Amplicon Denoising Algorithm
DNA	Deoxyribonucleic Acid
EPS	Extracellular Polysaccharide
HMME	Host Mediated Microbiome Engineering
HPRC	High Performance Research Computing
IAA	Indole-3-acetic Acid
ISR	Induced Systemic Resistance
ITS	Internal Transcribed Spacer
KEGG	Kyoto Encyclopedia of Genes and Genomes
LB	Lysogeny Broth
LSD	Least Significant Difference
LC	Liquid Chromatography
MAFFT	Multiple Alignment using Fast Fourier Transform
MS	Mass Spectrometry
NCBI	National Center for Biotechnology Information

NGS	Next Generation Sequencing
NTC	No Template Control
NTSI	Nearest Sequenced Taxon Index
OUT	Operational Taxonomic Unit
PBS	Phosphate Buffer Saline
PCoA	Principle Coordinates Analysis
PCR	Polymerase Chain Reaction
PERMANOVA	Permutational multivariate analysis of variance
PGPR	Plant Growth-Promoting Rhizobacteria
PICRUSt	Phylogenetic Investigation of Communities by Reconstruction of Unobserved States
QIIME2	Quantitative insights into Microbial Ecology 2
RDW	Root Dry Weight
rRNA	Ribosomal Ribonucleic Acid
RL	Root Length
ROS	Reactive Oxygen Species
RSA	Root System Architecture
RV	Root Volume
SA	Salicylic Acid
STAMP	Statistical Analysis of Metagenomic Profiles
SAR	Systemic Acquired Resistance
SRA	Sequence Read Archive

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Introduction

Global significance of water deficit

The world's population is anticipated to grow to over 8 billion people by 2030, entailing major issues for the agricultural sector with respect to food production and food security (Smol, 2012). Rice is the primary source of food for over half of the world's people and is considered to be the most important global crop (Khush, 2005). More than half of the world's rice is grown in Asia, amounting to over 50% of the caloric intake for the developing world (Khush, 2005). In addition to an increasing population, studies have shown there is tremendous vulnerability to climate change in the middle and high latitudes, where most of the rice in the world is produced (Rosenzweig and Parry, 1994). Water is the main limiting resource in global agricultural production, restricting harvests in approximately 70% of arable farmlands (Timmusk et al., 2014). Competition for freshwater supplies among agricultural, urban, and industrial water users has become more intense and is considered an imminent problem (Duriancik et al., 2008). Global climate change models indicate that many states in the western and south-central United States will experience increases in the frequency of extreme drought periods (Smit and Pilifosova). Some major concerns to urban, agricultural, and industrial sectors include water issues such as irrigation scheduling and efficiency, surface and groundwater management, and overall water quality. With increasing populations and decreasing water availability, these concerns are now extended to states that have previously had ample water resources. These human and climate driven changes in water

availability and quality could result in restriction in agricultural and urban landscape irrigation, which accounts for 62% of freshwater resource use in the United States (Kenny et al., 2009).

In Texas, it is predicted that population growth will outpace existing water supplies by 2060 (Scanlon et al., 2012). One-half of urban water use is devoted to irrigation of turfgrass, landscapes, and gardening, which largely relies on potable water supplies (Cabrera et al., 2013).Water scarcity and competition demand better options that can significantly conserve potable water resources. Conservative water uses coupled with drought resistant plant materials are one of the most sustainable approaches in urban landscapes and turf production.

From a national perspective, the most irrigated crop in the United States is turfgrass (Milesi et al., 2005). To date, landscape water conservation research efforts within the southern United States have primarily addressed development and use of native or more drought resistant plant materials, weather or sensor-guided irrigation technologies, deficit irrigation practices, and use of alternative water sources (Cabrera et al., 2013).

In Texas, it is estimated that the irrigation of home lawns, landscapes, and golf courses, of which turfgrass is the major component, accounts for nearly 47% of all water use within the urban/municipal water sector (Cabrera et al., 2013). As a result, turfgrass and landscape irrigation is the state's third largest water use, behind only agricultural irrigation and other urban uses.
The semi-arid climate conditions of El Paso, TX provide a constant selective pressure for tolerance to water deficiency on the grasses that survive in this environment and is therefore the basis for selecting this geolocation for bioprospecting microorganisms of interest (Chapter II).

Drought is a major challenge in the pursuit of increasing crop production (Vinocur and Altman, 2005). The four different categories of drought are meteorological, hydrological, socioeconomic, and agricultural, from which many different drought definitions exist across the

globe, varying with respect to severity, frequency, and economic impact (Ngumbi and Kloepper, 2016; Passioura, 2007). This research focused on the agricultural drought definition, defined as a period with declining soil moisture resulting in crop failure (Ngumbi and Kloepper, 2016; Passioura, 2007). There is increasing need for research in intensifying crop tolerance to withstand drought stress while simultaneously addressing the two major global challenges of water-related problems such as food security (Berg, 2009). Plants have evolved a variety of mechanisms to tolerate prolonged periods of water deficiency, including modification of phytohormone production (ex: abscisic acid) which trigger the activation of a multiple of drought stress response genes that can alter plant morphology (ex: leaf curling, increased allocation to promote root growth, etc.), manufacture reactive oxygen species (ROS) scavenging enzymes, manipulate the osmolality to maintain a negative water potential, and a variety of other drought tolerance based strategies (Chaves and Oliveira, 2004; Osakabe et al., 2014; Xiong et al., 2006). Plant adaptations to prolonged water deficiency can be characterized into the following four categories: escape, avoidance, tolerance, and recovery (Comas et al., 2013; Ngumbi and Kloepper, 2016; Passioura, 2007; Xiong et al., 2006; Xu et al., 2010). Drought escape is defined as a plant's capacity to end its normal life cycle prior to the onset of drought (Ngumbi and Kloepper, 2016; Xiong et al., 2006; Xu et al., 2010). Drought avoidance is the ability to maintain normal water status by mechanisms such as mitigating transpiration water loss through controlling stomatal closure or obtaining more water from the surrounding environment (Forni et al., 2017; Timmusk et al., 2014; Xu et al., 2010; Zhou et al., 2014). Drought tolerance is the continuation of normal growth and metabolism, despite the onset of drought conditions. These mechanisms include altering osmotic potentials, production of ROS scavenging enzymes to prolong membrane stability under dehydration, and the buildup of drought responsive secondary metabolites that function to stabilize plant tissue under

prolonged water deficit (Comas et al., 2013; Ngumbi and Kloepper, 2016; Passioura, 2007). Finally, the term drought resistance is seen as the combination of drought avoidance and drought tolerance (Blum, 2005).

Plant growth-promoting rhizobacteria

Research to increased water efficiency have focused on the development of novel crop water management technologies, plant breeding, and efforts to manipulate microbial ecology through the introduction of beneficial microorganisms that can aid the plant during times of water deficit. Plant growth-promoting rhizobacteria (PGPR) are beneficial microbes that live in the plant microbiome, or phytobiome, and are capable of increasing plant growth, development, and resistance under abiotic stress (Bresson et al., 2013; Dimkpa et al., 2009; Marulanda et al., 2009; Ngumbi and Kloepper, 2016; Timmusk et al., 2014). PGPR have been used to boost resistance to insect pests, fungal pathogens, and abiotic stressors such as heat and drought in cool-season turfgrass species such as tall fescue, perennial ryegrass, and fine fescues (Cheplick, 2007; Muller and Krauss, 2005; Saikkonen et al., 2006). Although many PGPR case studies have been well researched, the exact mechanisms behind this resistance remain largely elusive (Ngumbi and Kloepper, 2016). Possible explanations regarding the mechanism(s) associated with bacteria-induced drought resistance include findings that demonstrate that PGPR can produce different plant hormones, such as abscisic acid, auxins, gibberellins, cytokinins, and ethylene, which have the potential to increase plant growth, development, and resistance under abiotic stress (Yang et al., 2009). PGPR have also been shown to trigger induced systemic resistance (ISR) by bacterially produced compounds, which protect the roots from disease. ISR can be therefore be inversely correlated to drought stress susceptibility (Prime et al., 2006). It has also been documented that

certain rhizobacteria can increase ROS scavenging through antioxidant enzymes such as peroxidases and catalases, protecting host DNA and membranes from oxidative damage by eliminating free radicals in plants (Kohler et al., 2008). Perhaps most importantly, PGPR are capable of secreting bacterial biofilm in the form of an extracellular matrix, which can provide the plant with substantial macromolecules such as sugars and oligo-polysaccharides, contributing to numerous functions for plant growth and development (Dimkpa et al., 2009). Indeed, this naturally humectant extracellular biofilm matrix can directly improve water availability in the root medium through increased water retention, even in small concentrations (Chang et al., 2007). Some examples of PGPR mediated host abiotic stress tolerance includes alleviation of salt stress from strain *Achromobacter piechaudii* in tomato, drought tolerance from strains *Burkholderia phytofirmans* PsJN and *Enterobacter* sp. FD17 in maize, and drought tolerance from strains *Bacillus thuringiensis* AZP2 and *Paenibacillus polymyxa* B in wheat (Timmusk et al., 2014).

Plant microbiome

The plant microbiome, or phytobiome, is defined as the microbial communities that live in, on, and around a plant's above ground (phyllosphere) and below ground (rhizosphere). It is a key determining factor of plant health and productivity (Berendsen et al., 2012; Mueller and Sachs, 2015). The microbiome is a dynamic and intricately complex ecosystem, which has been a major challenge in research. Studies have shown that increased rhizosphere microbiota diversity is directly correlated to increased plant fitness, suggesting that the increased potential of multiple of interacting species yields greater adaptability and stability to disturbances (Lau and Lennon, 2012). However, other research has reported that reducing microbiome diversity does not necessarily lead to a decrease in ecosystem function, as other microbiota can occupy niches without affecting plant

productivity (Nannipieri et al., 2003). The complex challenge of connecting the microbiome influences with taxonomic changes led to the concept of a functional metagenome, which describes a microbiome based on functionality, rather than taxonomy (Nannipieri et al., 2003). This concept of a functional metagenome is closely associated with hologenome theory, which redefines the meaning of an individual as the combined collective genomes of the host and the genomes of the microbial communities (Zilber-Rosenberg and Rosenberg, 2008). The key to utilizing the microbiome community abundance and assemblage for enhancing agricultural production lies in understanding the microbiome functionality under different conditions.

The plant microbiome can influence a variety of beneficial effects such as increasing nutrient acquisition, enhancing disease management, and promoting tolerance to abiotic stress (Berendsen et al., 2012; Bulgarelli et al., 2013; Cook and Baker, 1983; Cook and Rovira, 1976; Lakshmanan et al., 2014; Mendes et al., 2013; Philippot et al., 2013). For instance, the microbiome plays a major role in disease-suppressive soils, in which the microbial community prevents the establishment or persistence of a pathogen (Berendsen et al., 2012; Bulgarelli et al., 2013; Cook and Baker, 1983; Cook and Rovira, 1976; Mendes et al., 2011; Schlatter et al., 2017). In a phosphorus deficient environment, microbiomes containing mycorrhizae can enhance soil P solubilization by releasing of organic acids like oxalate (Berendsen et al., 2012; Bolan, 1991), therein increasing cation exchange capacity (CEC). The microbiome can also contribute significant increases in drought stress tolerance through the alteration of root system architecture, exuding molecules like 1-aminocyclopropane-1-carboxylate (ACC) deaminase, abscisic acid and salicylic acid involved in plant stress response, and reducing reactive oxygen species (ROS) (Naylor and Coleman-Derr, 2017).

There are a variety of mechanisms that can influence the diversity and overall abundance of bacteria and fungi in a rhizosphere microbiome. Rhizodeposition of carbon from root exudates is generally regarded as the largest contributing factor in determining microbial abundance and profiling. Through the deposition of carbon in root exudates, plants are able to manage the type and overall abundance of microbiota in the rhizobiome. In addition to concentration, plants can also manipulate the specific type of carbon available in the rhizosphere, effectively using root exudates as a chemoattractant to selectively recruit specific taxa that are able to metabolize these exudates (ex: malate, citrate, oxalate, etc.), and influence the microbiome community composition. This mechanism is a product of coevolution between mutualistic microbiota and the host. The degree of specificity with respect to the type of carbon exudates, molecular communication for plant-microbe signaling, and root physical adhesion morphology increases as the symbiont and the host coevolve (Berendsen et al., 2012; Kinkel et al., 2011; Philippot et al., 2013). It is for this reason that the host is the largest contributing factor in determining rhizobiome community composition (Berg, 2009).

Other conditions such as soil type, seasonality, host developmental stage, and perturbation disturbances (prolonged water deficit, management practices, etc.) (Bakker et al., 2012; Bulluck et al., 2002; Chaparro et al., 2013) can have a profound effect on microbiome community composition. For example, *Arabidopsis thaliana* studied at four different timepoints revealed significant differences in rhizosphere bacterial community composition during the seedling stage compared to the vegetative, bolting, or flowering stages (Chaparro et al., 2013). Soil properties (structure, texture, pH, nutrient content, etc.) have been shown to affect microbial abundance, and play a greater deterministic factor than geospatial scales (Lareen et al., 2016). Seasonal impacts have been shown to have influence taxonomic and functional community succession events in the

phyllosphere microbiome in common bean, soybean, and canola (Copeland et al., 2015). Organic soil amendments (composted cotton-gin trash, composted yard waste, or cattle manure) have been shown to impact the maize and melon rhizobiome by increasing microbial heterogeneity, decreasing evenness, and shifting the soil microbiota to a higher abundance of biocontrol fungi (*Trichoderma* spp.), lower abundances of pathogens (*Phytopthora* spp. and *Pythium* spp.), and higher concentrations of Ca^{2+}, K^{+}, Mg^{2+}, and Mn^{2+} (Bulluck et al., 2002) when compared to a conventional farming environment using mineral fertilizers. A study of crop rotation (winter wheat and grass-clover) cropping systems over two decades showed soil microbiomes amended with organic fertilizers shifted microbial taxa that promoted beneficial microbes, pathogen suppression, and contained specific microbial guilds associated with the degradation of complex organic compounds (Hartmann et al., 2015).

Goal

The overarching goal of my research is to improve our understanding of the ecological and evolutionary implications of plant-microbe interactions in a water deficient environment, with potential outcomes that can be directly used for the alleviation of drought stress in turfgrass and cereal crop production.

Specific Objectives

Objective 1: Conduct a bioprospecting screen of grass rhizospheres from a semi-arid environment in order to isolate plant growth promoting rhizobacteria that can rapidly colonize a seedling rhizosphere and mediate drought stress in multiple cereal hosts.

Objective 2: Use host-mediated microbiome engineering to design a microbial community that increases drought tolerance in wheat seedlings.

Hypothesis

Hypothesis 1: PGPR that can rapidly colonize the seedling rhizosphere and confer drought tolerance can be isolated from a semi-arid environment in multiple hosts.

Hypothesis 2: Host mediated microbiome engineering can be used to alter a rhizosphere microbiome to confer drought resistance.

CHAPTER II

BIOPROSPECTED RHIZOBACTERIA ENHANCE DROUGHT TOLERANCE IN GRASSES

Synopsis

This study identified two novel plant growth-promoting rhizobacteria (PGPR) strains, which when inoculated into the rhizospheres of wheat (*Triticum aestivum*) and maize (*Zea mays*) seedlings, resulted in statistically significant delays in the onset of drought symptoms. These isolates were identified through the application of a bioprospecting screen designed to isolate PGPR capable of rapidly colonizing the rhizosphere and mediating drought stress in multiple hosts. This phenotype was associated with alterations in root system architecture consistent with mitigating drought stress. In wheat, both PGPR treatments significantly increased root branching, and *Bacillus* sp. (12D6) significantly increased root length, when compared to the control ($P \leq 0.05$). In maize, both PGPR treatments significantly increased root length, root surface area, and tips when compared to the control ($P \leq 0.05$), and *Enterobacter* sp. (16i) exhibited greater effects in root length, diameter, and branching when compared to *Bacillus* sp. (12D6) or control. LC-MS based phytohormone profiling of PGPR centrifuged pellets and filtrates demonstrated that both strains produced indole-3-acetic acid (IAA) and salicylic acid (SA) *in vitro*. Importantly, the effects of PGPR inoculation occurred concurrently with the onset of water deficit, demonstrating the potential of the PGPR identified from this bioprospecting pipeline for use in production systems without requiring seed coat application or a long colonization period.

Introduction

Drought is a major abiotic stress threatening agricultural production worldwide. In the last forty years, drought stress has reduced yields in cereals by as much as 10% (Lesk et al., 2016) and is forecasted to affect production on over 50% of the arable land by 2050 (Vinocur and Altman, 2005). In order to address this global challenge in agriculture, research has focused on improving germplasm and developing crop management practices to increase water use efficiency (Ngumbi and Kloepper, 2016; Passioura, 2007). However, recent attention has turned to the application of beneficial microorganisms that mediate drought tolerance and improve plant water-use efficiency and these efforts have been augmented due to technological advances in next generation sequencing and microbiomics (Dimkpa et al., 2009; Marulanda et al., 2009; Ngumbi and Kloepper, 2016; Vurukonda et al., 2016; Yang et al., 2009).

The application of plant growth-promoting rhizobacteria (PGPR) is considered a sustainable synergistic biological approach to cope with water deficiency in crop production. PGPR readily colonize the root rhizosphere and establish both free-living and intimate associations with host plants. Often, these interactions lead to enhancement of crop productivity and mitigation of biotic and abiotic stresses through a variety of mechanisms (Barnawal et al., 2017; Berg, 2009; Dimkpa et al., 2009; Forni et al., 2017; Gontia-Mishra et al., 2016; Liu et al., 2013; Mayak et al., 2004; Mendes et al., 2013; Ngumbi and Kloepper, 2016; Porcel et al., 2014; Vacheron et al., 2013; Vurukonda et al., 2016). PGPR may play critical roles as suppressors of plant disease, biofertilizers, alleviators of abiotic stress, and remediators of toxins from the soil (Mayak et al., 2004; Naveed et al., 2014; Timmusk et al., 2014). Mechanisms associated with PGPR-derived drought tolerance include alterations in host root system architecture, osmoregulation, management of oxidative stress via the biosynthesis and metabolism of phytohormones or the

production of antioxidants for scavenging reactive oxygen species (ROS), the production of large chain extracellular polysaccharide (EPS) that may serve as humectant, and transcriptional regulation of host stress response genes (Barnawal et al., 2017; Dimkpa et al., 2009; Forni et al., 2017; Gontia-Mishra et al., 2016; Liu et al., 2013; Ngumbi and Kloepper, 2016; Osakabe et al., 2014; Timmusk et al., 2014; Vacheron et al., 2013; Vurukonda et al., 2016).

The objective of this study was to isolate PGPR capable of rapidly colonizing seedling rhizospheres and mediating drought stress in multiple cereal hosts. For this purpose, a bioprospecting screening method was developed that emphasized the following:

a) a selection of a likely source containing PGPR

b) a pre-screening process focused on desired plant phenotypes

c) a final screening process focused on candidates likely to provide desired outcomes under practical production practices on both wheat and maize.

The original source of PGPR were the rhizospheres of perennial grasses collected from El Paso, TX, where the semi-arid environment provides a strong selective pressure for survival under nearly constant water deficit. The rationale for choosing the starting material was that perennial grasses growing vigorously under pervasive water stress conditions were likely to foster a microbiome capable of mediating drought stress. The pre-screening process focused on the desired host phenotype, rather than bacterial phenotypes. The host phenotype used for screening was the delayed of onset of drought stress symptoms in seedlings, since seedling establishment is often the most vulnerable stage and may have large impacts on crop stand and yield (Pessarakli, 1999). The final selection process focused on the identification of PGPR that are most likely to have applications in existing commercial production systems. Given current limitations in “seed space” for new growth stimulating products combined with the difficulties in reliable formulation of

application-friendly seed treatments, the focus of this study was on identifying isolates that could be applied as needed prior to the onset of water stress conditions. The screening protocol was designed to specifically select isolates that could rapidly colonize and provide benefits to the host, e.g. if inoculated at the onset of water deficit conditions. In this manner, this screen provides the unique ability to select strains that can be added as needed, as compared to current seed coating applications. Candidate PGPR strains demonstrating robust effectiveness were identified by screening isolates on two different hosts.

Materials and methods

Rhizobacteria sampling and high throughput screening

Twenty-five bermudagrass thatch core samples (10 cm in diameter and 15 cm in depth) were collected in the summer of 2015 and 2016 in El Paso, Texas, USA (Figure 1A). Sampling sites included medians, parks, roadsides, and ranches. Intact core samples were immediately shipped upon removal under ambient temperatures to College Station, TX. Each sample core was then subdivided into 2-inch diameter cores, transferred to a round plastic pot (10 cm diameter, 12 cm height), filled-in with sterilized potting mix (Metro-Mix 900, Sun Gro Horticulture, Agawam, MA), and grown in a greenhouse for 14 days. Grasses were exposed to three different levels of watering: non-stressed (watering up to the field capacity every other day), moderate stress (watering once a week), and severe stress (no watering) (Figure 1B). The onset of drought symptoms was monitored and recorded based on phenotype: leaf wilting, curling, tip burning, and plant lodging. The five cores containing plants for which drought symptoms were most delayed under both the moderate and severe watering regimes were used for the next step: bacterial isolation and preservation for screening trials.

Rhizosphere samples for bacterial isolation were obtained from one gram of root tissue, excised from the grasses in each of the selected cores. Root tissue samples were first washed in sterile dH_2O to remove detritus and non-root adherent soil, suspended in 10 ml of 0.1 M phosphate buffered saline (1 min), and macerated using a drill homogenizer (115V Bio-Gen PRO200 homogenizer unit, 5 mm x 75 mm generator probe). PBS suspensions were serially diluted and plated on Luria-Bertani (LB) agar amended with 5 mg L^{-1} cycloheximide and 10% sorbitol (Kavamura et al., 2013). Plates were maintained 25ºC and inspected daily for bacterial growth. Morphologically distinct colonies were re-isolated to obtain axenic cultures and then grown separately overnight in LB broth (25ºC, 120 rpm agitation) and stored in 40% glycerol at -80ºC.

PGPR screening

Wheat (*Triticum aestivum* subsp. *aestivum* cultivar TAM111) and Maize (*Zea Mays* cultivar B73) seeds were surface sterilized in 10% NaOCl for 10 min, followed by 10 subsequent rinses in sterile dH_2O. Seeds were germinated on sterile filter paper 24 hr. at 37ºC for wheat and 25ºC for maize. Germinated seeds were planted separately in pots (10 cm diameter, 12 cm height) containing 400 g sterilized Metro-Mix 900. Seedlings were watered to field capacity every day and cultivated in a growth chamber for 7 days (30ºC, using fluorescent bulbs emitting 300 µmol m^{-2} s^{-1}, 12 hr. :12 hr. light / dark cycle). Plants were inoculated 7 days post germination with test strains, followed by withholding water for the next 7 days. For the bacterial inoculum, overnight cultures were grown in LB at 25ºC, collected via centrifugation (2,500 x g, 5 min) and re-suspended in an equal volume of 0.1 M PBS. 80 µl of resuspended inoculum was applied to the soil at the base of each seedling. Inoculation with 0.1 M PBS was used as a no-inoculum control. For isolates that demonstrated PGPR activity, in subsequent trials inoculum densities were regulated to insure

populations of approximately 10^7 CFU/ml via optical density (600 nm) measurements. Growth curves comparing colony counts and optical density were used to determine the optical densities that provided the desired population densities.

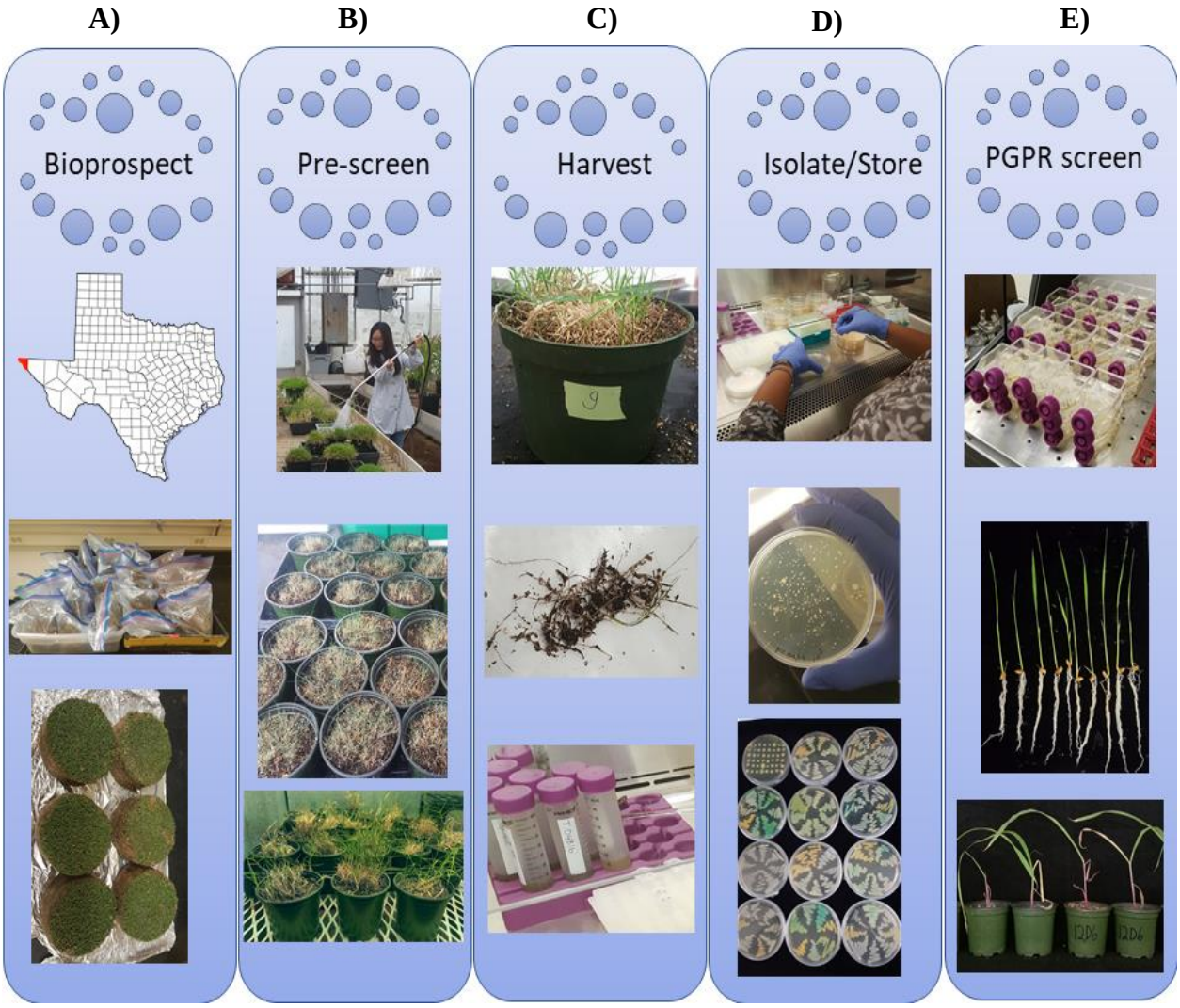

Figure 1 PGPR Bioprospecting workflow. A) Grass thatch cores were removed from the semiarid environment of El Paso, Tx. B) Subcores were exposed to water deficit in a greenhouse and selected based on host phenotype (delayed onset of drought symptoms). C) Root tissue samples from grasses in five of the selected cores were macerated and resuspended 0.1M PBS. D) The PBS solution was serially diluted and plated on LB + cycloheximide or sorbitol. Morphologically distinct colonies were isolated, re-streaked for single colonies and preserved in glycerol for long term storage. E) LB overnights of each isolate were then used as inoculant for wheat and maize 7 days post germination. At the time of inoculation, plants were exposed to water deficit by withholding water, and screened for delayed of onset of drought symptoms relative to non-inoculated controls.

Drought tolerance phenotyping

At the end of the 7-day water stress treatment (14 days post planting), inoculated and non-inoculated plants were examined for drought symptoms (e.g., wilting, leaf curling, marginal leaf necrosis). Plants were then removed from the soil, with special care to preserve the intact root system. Roots were washed to remove soil and detritus via spraying with dH_2O against a 0.5 mm mesh sieve. Harvested root and shoot tissues were saturated with dH_2O via storage in wet germination paper at 4ºC overnight, in preparation for downstream analysis (Himmelbauer et al., 2004). Washed roots were separated from above ground tissue, submerged in dH_2O and spread out to prevent overlap in a root positioning tray (20 × 30 cm) with three roots per tray. Roots were scanned using a flatbed scanner (EPSON, Perfection V-750). Root image data obtained by scanning were analyzed using WinRHIZO Arabidopsis 2017a (Regent Instruments Inc., Quebec, Canada, 2000), generating estimates of total root length, root surface area, average root diameter, number of root tips, and root branching as previously described (Arsenault et al., 1995; Himmelbauer et al., 2004). For plants that exhibited delayed drought stress symptoms relative to control plants, bacterial population sizes were determined via serial dilution plating. In all experiments, root population sizes were 10^6 to 10^7 CFU / g of rhizosphere, defined as root and root adherent soil. Bacteria were re-isolated from roots on LB amended with cycloheximide and stored as before.

Isolate sequencing

For bacterial strains of interest, taxonomic information was obtained via sequencing of the 16S and 23S ribosomal RNA subunit and ITS regions (Dinesh et al., 2015; Stackebrandt and Goebel, 1994). Genomic DNA from each strain was extracted using the CTAB protocol (William

et al., 2012). Polymerase chain reaction (PCR) was used to amplify the target region with the following primers: 16S region forward 8F/pA (5′-GAGTTTGATCCTGGCTCAG-3′) and 23s reverse p23SR01 (5′-GCTGCTTCTAAGCCAAC-3′) (Dinesh et al., 2015; Stackebrandt and Goebel, 1994). PCR was performed in a thermocycler (Applied Biosystems® Thermocycler 2720) with the following reaction conditions: 1 min 95°C; 35 cycles of 1 min 95°C, 1 min 52.7°C, and 1.5 min 72°C; 1 cycle 10 min 72°C; maintain at 4°C until retrieval. PCR amplicons were gel purified using the Wizard® SV Gel and PCR Clean-Up System (Promega Corporation), and sequenced (Sanger sequencing, Eton Biosciences) with the aforementioned PCR primers and sequencing primers 1542R/pHr (5′-TGCGGCTGGATCACCTCCTT-3′) and 1542R/pH (5′-AAGGAGGTGATCCAGCCGCA-3′). The reads were aligned using MAFFT algorithm in Benchling (https://benchling.com). Consensus alignments were taxonomically identified at the genus level via NCBI nucleotide Basic Local Alignment Search Tool (BLASTN) (https://blast.ncbi.nlm.nih.gov/).

Phytohormone profiling

Ten milliliters of LB overnight cultures from each strain were pelleted via centrifugation (2500 x g, 10 min). Cell free supernatants were obtained using a Nalgene® Rapid-Flow™ sterilization filter (0.2 µm nitrate cellulose membrane) and vacuum infiltration. Pellet and filtrate samples were lyophilized for 24 hr. followed by resuspension in 500 µl extraction buffer consisting of n-propanol/H_2O/ HCl (2:1:0.002 by volume) spiked with 500 nM of the following deuterated internal standards: d-ABA ([2H6] (+)-cis,trans- abscisic acid; Olchemlm cat# 0342721), d-ACC (1-Aminocyclopropane-2,2,3,3-d4-carboxylic acid; Sigma cat#736260), d-trans-Cinnamic acid (d7- cinnamic acid; Sigma cat#513954), d-IAA([2H5] indole-3- acetic acid; Olchemlm cat#

0311531), d-JA (2,4,4-d3; acetyl-2,2-d2 jasmonic acid; CDN Isotopes cat# D-6936), d-SA (d6-salicylic acid; Sigma cat#616796). Following resuspension, phase separation was conducted via the addition of dicholormethane (CH_2Cl_2) for 30 min at 4°C, followed by centrifugation (21,255 x g, 10 min). The organic phase was removed, evaporated under N_2 gas, re-solubilized in 150 µl methanol, and incubated overnight at -20°C. Samples were then centrifuged (21,255 x g, 5 min). After centrifugation, 10 µl of supernatant from each sample were injected into a C18 analytical column for liquid chromatography analyte separation, followed by detection via triple quadruple mass spectrometry. Target phytohormones in samples were quantified for via comparison against the internal deuterated standards as previously described (Strauch et al., 2015; Stumpe et al., 2005).

Statistical analysis

PGPR screening assays were conducted in a completely randomized block design with five replications. Final assays were repeated once. Plant phenotype data from WinRHIZO and LC-MS results were analyzed using an analysis of variance (ANOVA) (SAS version 9.3 software, SAS Institute Inc., Cary, NC). Pairwise comparisons between the treatments were conducted using Fischer's least significant difference (LSD) test [$P \leq 0.05$]. All scripts used in SAS analysis can be found in Appendix B.

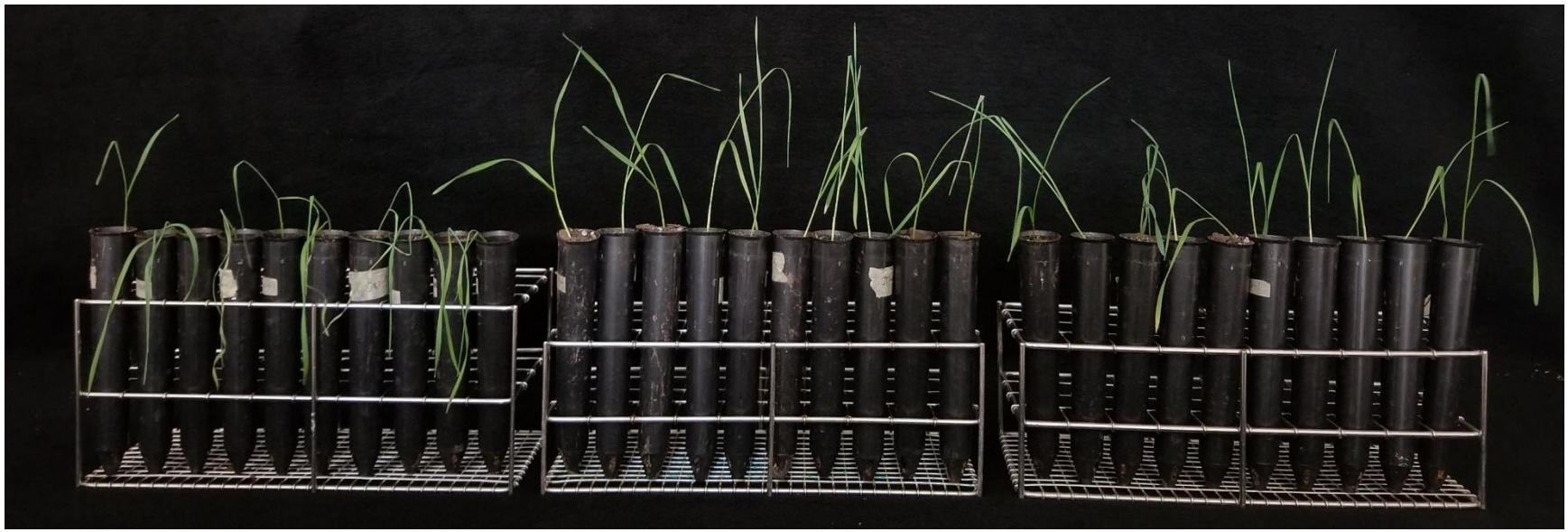

Figure 2 Image of wheat seedlings. Plant growth promoting rhizobacteria (PGPR) *Bacillus* sp. (12D6) (middle) and *Enterobacter* sp. (16i) (right) demonstrating delayed onset of water deficit signs versus control (left) in wheat seedlings after exposure to 10 days of continuous water deficit.

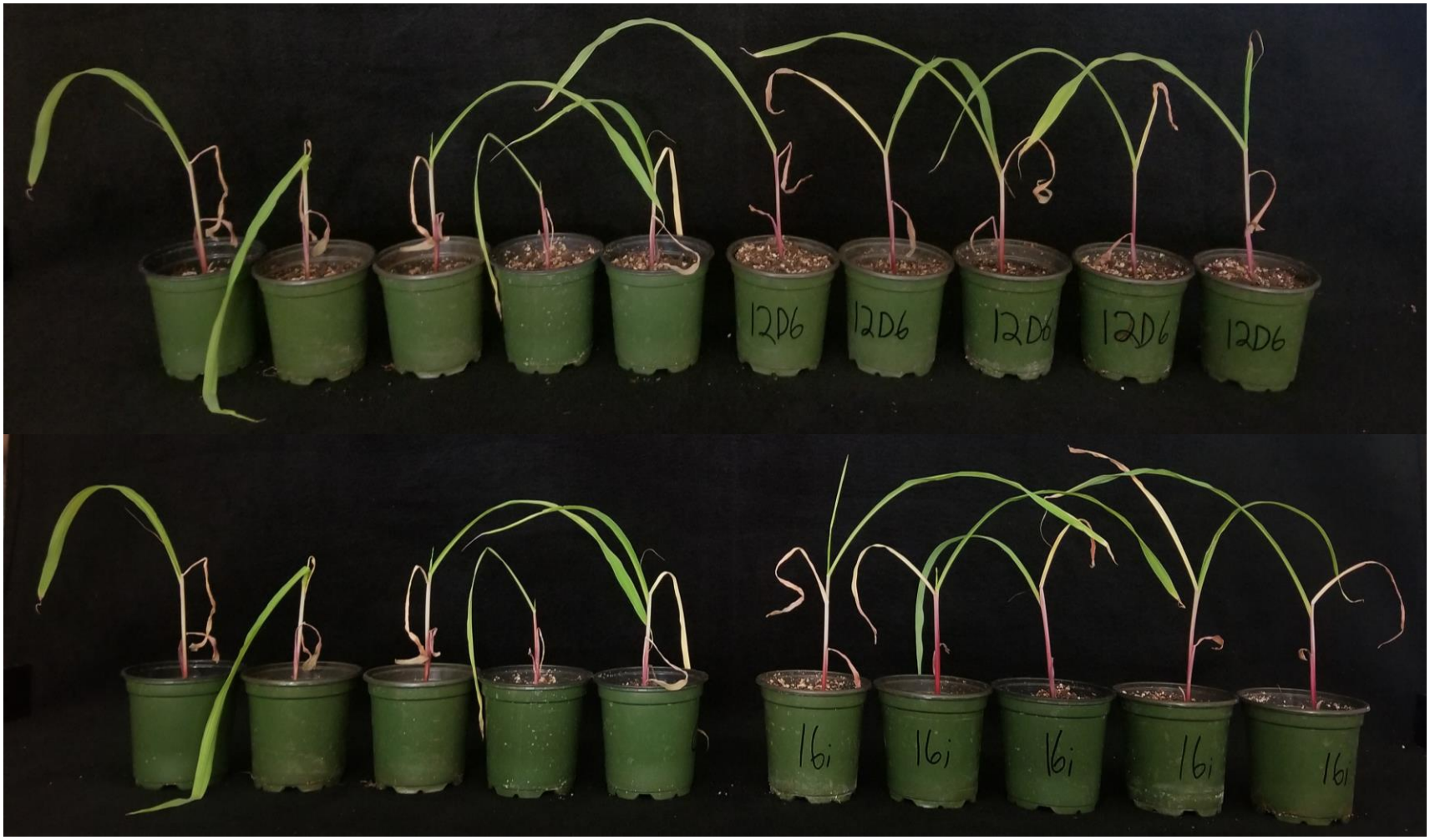

Figure 3 Images of maize seedlings. Plant growth promoting rhizobacteria (PGPR) *Bacillus* sp. (12D6) (top right) and *Enterobacter* sp. (16i) (bottom right) demonstrating the delayed onset of water deficit in maize seedlings versus control (top and bottom left) after exposure to 10 days of continuous water deficit.

Results

PGPR screening and Drought tolerance phenotyping

Out of 200 tested isolates, soil inoculation by two PGPR strains, 12D6 and 16i, significantly alleviated drought stress symptoms in both wheat and maize seedlings (Figure 2 & Figure 3). Qualitative assessment of plant performance across replicate experiments suggested strain 12D6 was somewhat more effective in mediating a delay in the onset of drought symptoms in wheat, whereas strain 16i was more effective in mediating this effect maize. Results from the NCBI BLASTN query based on rRNA sequence identified strain 12D6 (accession no. MH678658 and MH683042) as *Bacillus* sp. (ident = 99%) and 16i (accession no. MH678659 and MH683043) as *Enterobacter* sp. (ident= 99%).

Table 1 PGPR Analysis of variance (ANOVA). The effect of PGPR treatment on wheat and maize root systems following a 7-day water deficit.

Dependent Variable	*Triticum aestivum*				*Zea Mays*			
	df	Mean squared	*F*	*P*	df	Mean squared	*F*	*P*
Root Length	2	4512.80756	3.13	0.0599	2	26904.8926	13.89	<0.0001
Root Surface Area	2	23.16365397	1.93	0.1653	2	147.012501	4.48	0.0198
Average Diameter	2	0.00067100	1.29	0.2929	2	0.00723170	8.82	0.0010
Root Tips	2	69630.700	1.42	0.2596	2	207948.394	5.02	0.0132
Root Branching	2	110906.8000	4.91	0.0152	2	512832.212	8.72	0.0010

Table 2 Pairwise comparisons using Fischer's LSD test (n=10) of A. Wheat and B. Maize root system architecture with and without PGPR inoculation, analyzed using WinRHIZO software. In wheat, both PGPR treatments significantly increased root branching, and *Bacillus* sp. (12D6) significantly increased root length, when compared to the control ($P \leq 0.05$). In maize, both PGPR treatments significantly increased root length, root surface area, and tips when compared to the control ($P \leq 0.05$), and *Enterobacter* sp. (16i) exhibited greater effects in root length, diameter, and branching when compared to *Bacillus* sp. (12D6) or control.

A. Wheat

Wheat	Root Length (cm)	Root Surface Area (cm^2)	Average Diameter (mm)	Root Tips	Root Branching
Bacillus sp. 12D6	165.40 A	11.88	0.248	676.2	604.6 A
Enterobacter sp. 16i	161.49 AB	12.08	0.236	628.3	544.8 A
Control	126.81 B	9.35	0.233	513.8	399.8 B

B. Maize

Maize	Root Length (cm)	Root Surface Area (cm^2)	Average Diameter (mm)	Root Tips	Root Branching
Bacillus sp. 12D6	323.94 B	40.49 A	0.399 B	1149.8 A	1299.6 B
Enterobacter sp. 16i	370.16 A	42.55 A	0.367 A	1098.2 A	1600.4 A
Control	271.31 C	35.44 B	0.417 B	890.1 B	1181.6 B

Analysis of root architecture

Results from a two-way ANOVA (host X bacterial treatment) revealed that given the larger size of the maize root system compared to the wheat root system, all maize root system dependent variables were statistically larger than those of wheat ($P < 0.0001$). Consequently, the ANOVA was performed separately for each host (Table 1).

In wheat, the root systems of seedlings treated with either bacterial inoculum were more branched than those of the non-inoculated seedlings. However, treatment of seedlings with the *Bacillus* sp. (12D6) inoculum also contributed to greater total root length compared to the control treatment (Table 2). In maize, the root systems of seedlings treated with either bacterial inoculum were larger in terms of total root length and surface area and had more root tips than non-inoculated seedlings (Table 2). Some differences between the treatments in other metrics were observed. The root systems of seedlings treated with *Enterobacter* sp. (16i) were larger in terms of total root length and more branched, but roots had a smaller average diameter than those of the *Bacillus* sp. 12D6-treated seedlings or the controls (Table 2).

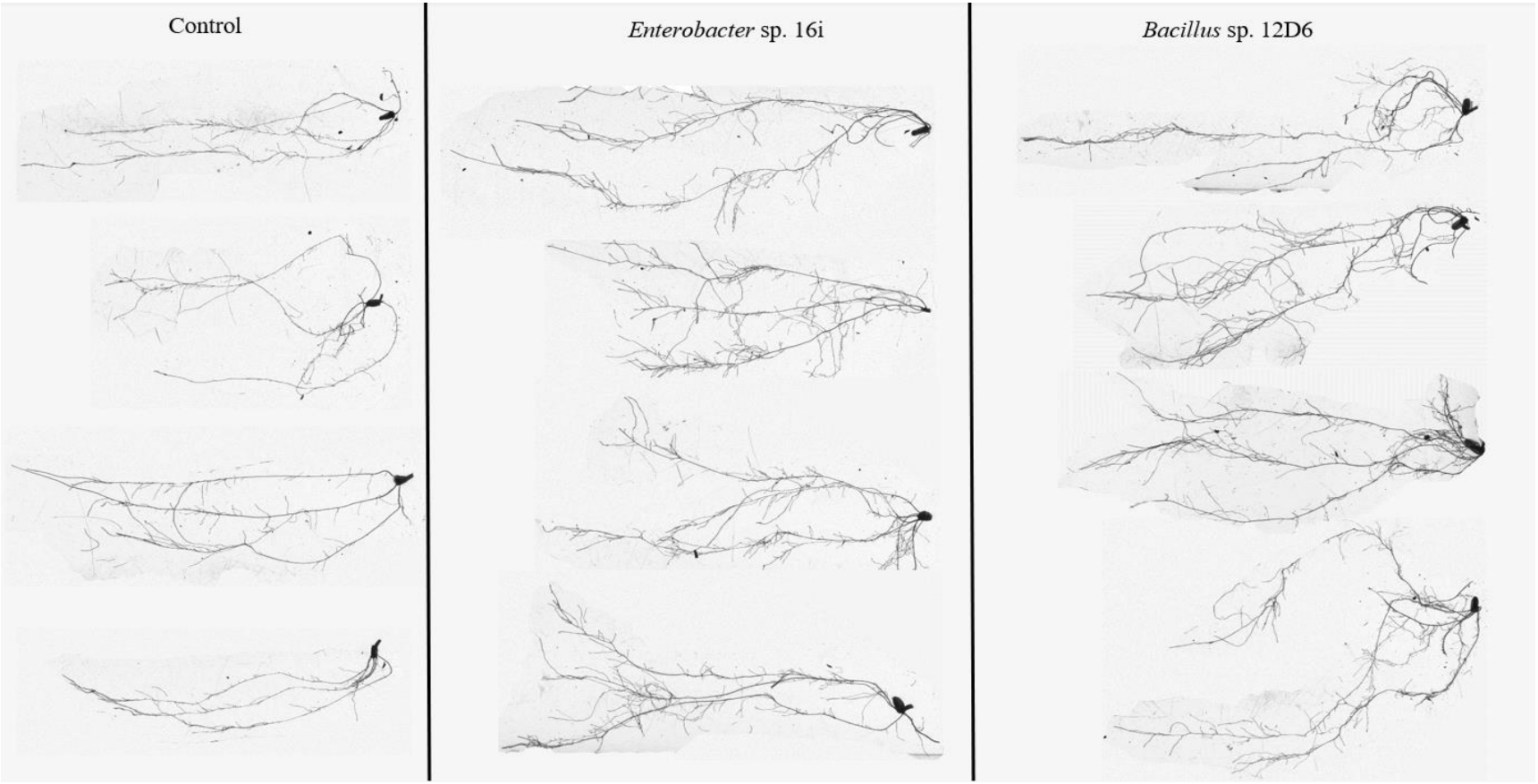

Figure 4 Root system architecture in wheat seedlings. Comparisons of image scans of wheat seedlings treated with the control (left), *Enterobacter* sp. (16i) (center) and *Bacillus* sp. (12D6) (right) after exposure to 10 days of continuous water deficit.

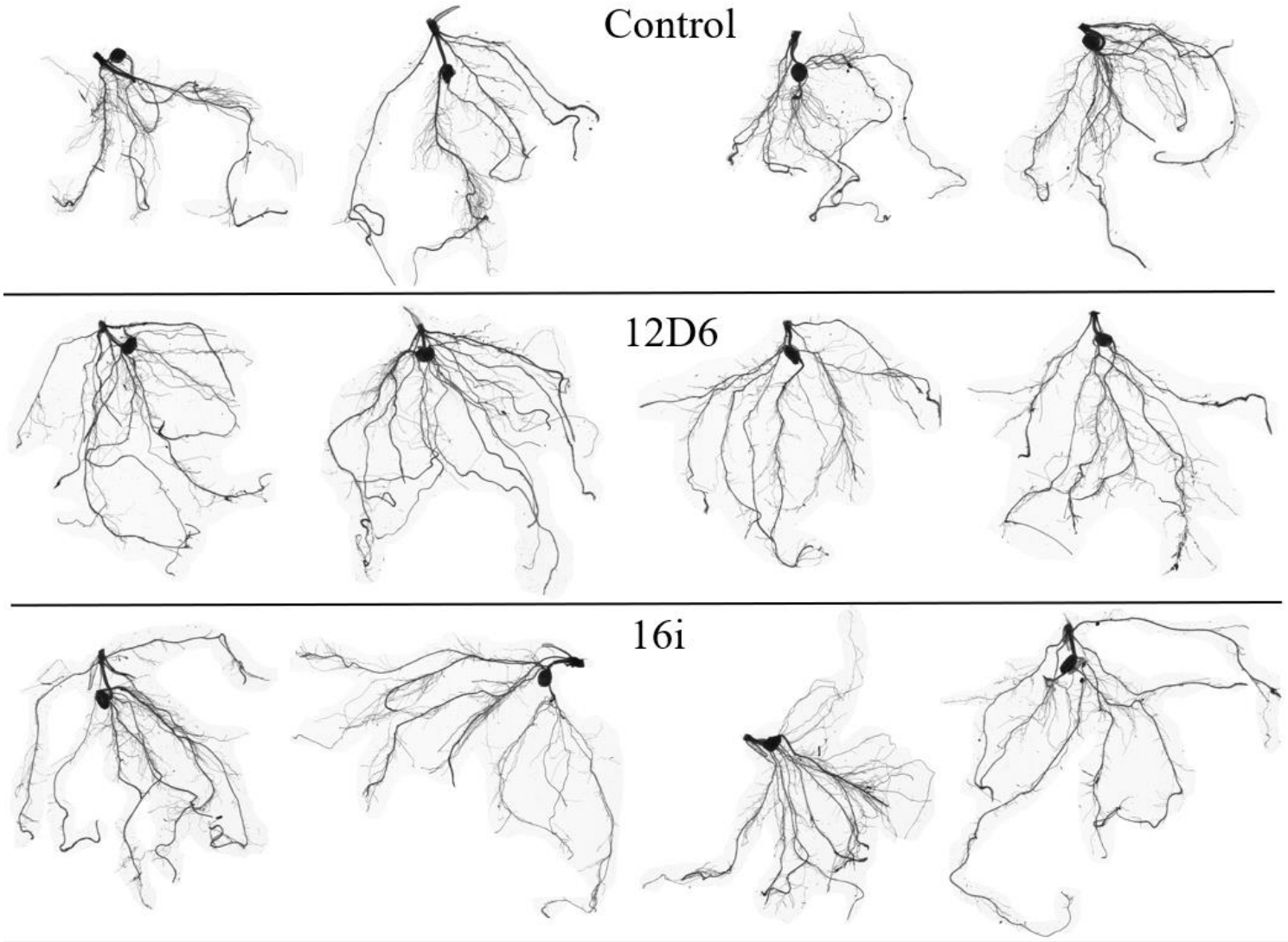

Figure 5 Root system architecture in maize seedlings. Comparisons in image scans of maize seedlings treated with the control (left), *Enterobacter* sp. (16i) (center) and *Bacillus* sp. (12D6) (right) after exposure to 10 days of continuous water deficit

Phytohormone profiling

Targeted analyte LC/MS based phytohormone profiling of PGPR strains grown *in vitro* revealed that both strains produced indole-3-acetic acid (IAA) (Figure 6) and salicylic acid (SA) (Figure 7) in relatively high amounts ($P < 0.0005$) compared to the other phytohormones profiled and the LB control ($P < 0.0001$). The analytes were found both in the pelleted cells and the filtrate compared to the LB control, indicating both PGPR strains may secrete both compounds.

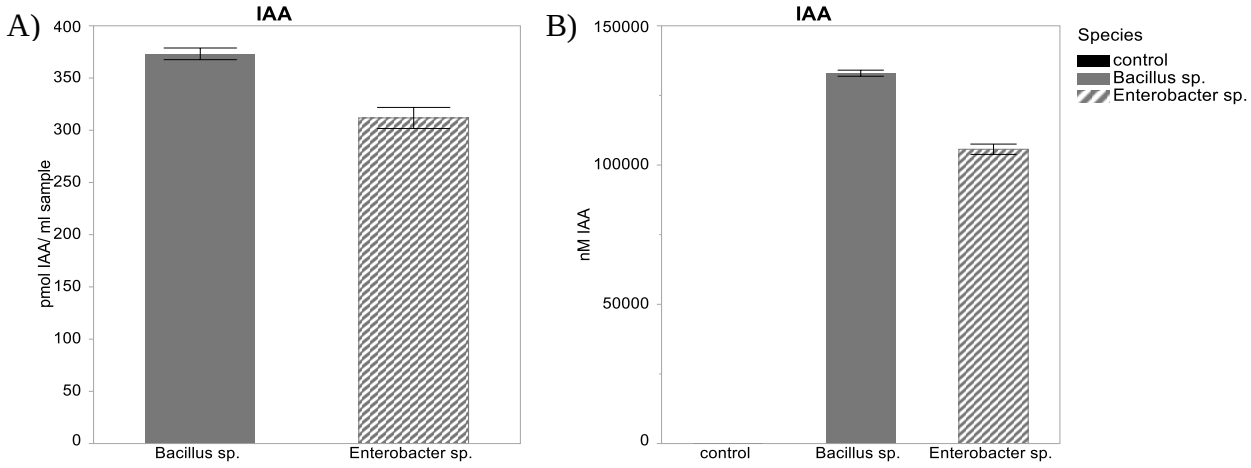

Figure 6 Indole-3-acetic acid (IAA) concentrations. *In vitro* IAA produced in the pellet (left) and the filtrate (right) in vitro in LB overnight cultures

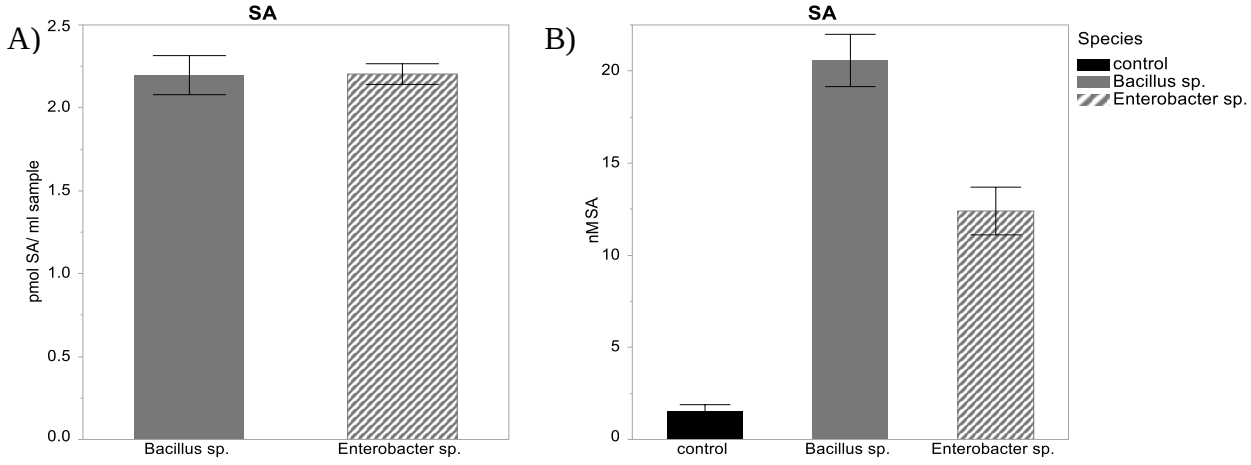

Figure 7 Salicylic acid (SA) concentrations**.** *In vitro* SA produced in the pellet (left) and the filtrate (right) in vitro in LB overnight cultures

Discussion

This study reports the development and use of a bioprospecting pipeline to effectively screen PGPR isolates for the ability to rapidly mediate plant drought stress symptoms in multiple cereal hosts when applied to plants under water deficit conditions. By starting with samples of perennial grasses that appeared healthy under constant water deficit conditions in the semi-arid environment of El Paso, TX, we attempted to focus in on rhizosphere microbiomes that may be selected for and adapted to mediating drought tolerance to grasses under these conditions. The pre-screening approach was based on selection of PGPR that mediated the desired seedling phenotype of delayed onset and severity of drought symptoms. Application of the PGPR to the base of plants at the onset of drought stress selected for specific PGPR capable of producing these results rapidly and under water stress conditions.

Using this pipeline two PGPR strains were identified: *Bacillus* sp. (12D6) and *Enterobacter* sp. (16i). Both wheat and maize seedlings experienced a delay in the onset of drought symptoms when treated with either isolate, although visual assessment of plant performance suggested strain 12D6 was somewhat more effective in mediating drought symptoms in wheat, whereas strain 16i was more effective in maize. These phenotypic differences in seedling tolerance of drought stress were associated with changes in root system architecture, although again there were some differences between hosts in response to the two strains. For instance, in wheat, although both strains had a significant effect on root system architecture, producing more branched root systems than non-inoculated seedlings, 12D6- treated seedlings also produced larger root systems in terms of total root length than 16i-treated seedlings or the controls. In maize, both strains produced root systems that were larger in terms of total root length and surface area and had more root tips than non-inoculated seedlings. However, the root systems of 16i-treated seedlings also had greater total

root length, more branches, and smaller average root diameters than those of 12D6-treated seedlings or the controls. The production of greater linear root length, surface area, and more root tips has been correlated previously with better water stress tolerance and overall improvements in maintaining plant productivity under drought (Comas et al., 2013). Root system length and surface area contribute to better soil exploration, whereas the proliferation of higher order roots resulting in more root tips are important for root water uptake capacity (Barnawal et al., 2017; Blum, 2005; Naseem and Bano, 2014; Ngumbi and Kloepper, 2016; Vardharajula et al., 2011). Previous research demonstrates that reductions in root diameter may enable faster relative growth rates and rapid resource acquisition through expansion of the root system coupled with lower investment in dry biomass (Birouste et al., 2014; Garnier, 1992; Wahl and Ryser, 2000). Although hosts differed somewhat in how their root systems responded to bacterial treatment, in general these results suggest that water stress tolerance resulted in part from bacterially-mediated changes in root system architecture that may have led to enhanced avoidance of drought stress symptoms.

Previous research suggests that host-specific selection of and response to PGPR are complex (Drogue et al., 2012; Kloepper, 1996; Smith and Goodman, 1999). Indeed, differences in the response of spring wheat to *Bacillus* spp. at the cultivar level have been observed previously (Chanway et al., 1988). At the molecular level, plant-microbe specificity may be driven by plant and microbial signals important for host-microbe perception, microbial recruitment, and microbial initiation of host response to symbiosis traits (Smith and Goodman, 1999). In the case of drought stress-mediating PGPR, bacterial adaptation to water stress (e.g. extracellular polysaccharide production), and host specific responses to drought stress (e.g. root system architecture, stomatal closure) also may be important. Success in mediating water stress tolerance by PGPR ultimately depends on effective root colonization, reliable expression of microbial traits important for PGPR

activity, and cultivar specific differences in mechanisms of adaptation to drought stress (Drogue et al., 2012; Kloepper, 1996). Although both strains successfully colonized the rhizosphere at concentrations of at least 10^6 CFU / g root sample (root and rhizosphere soil), any of these other factors may have contributed to the observed differences in the effectiveness 12D6 and 16i in mediating drought stress in maize and wheat.

Production and secretion of bacterial compounds that may serve as stimulators of plant growth and development (e.g. phytohormones) or signals (e.g. salicylic acid, indole-3-acetic acid) within whole-plant signaling pathways have been reported to be involved in bacterially-mediated drought tolerance in plants (Bakker et al., 2014; Dodd et al., 2010; Ngumbi and Kloepper, 2016). In this study, LC-MS based profiling of bacterially produced compounds demonstrated that both of these bacterial strains produce indole-3-acetic acid (IAA) and salicylic acid (SA) when grown *in vitro* LB liquid overnight cultures. Moreover, these compounds were detected both in the cellular and supernatant fractions, indicating they may be secreted.

Bacteria have multiple pathways for IAA biosynthesis, which may function in tryptophan storage, and regulation of tryptophan-dependent IAA biosynthesis may have wide-spread effects on bacterial gene expression patterns (Duca et al., 2014; Spaepen and Vanderleyden, 2011; Spaepen et al., 2007). Research has shown that that bacterially produced IAA may function in microbe-microbe signaling and is important for establishing symbiotic relationships with plants, such as during nodule or tumor formation (Spaepen et al., 2007). It is presumed that over 80% of all bacteria isolated from the rhizosphere can produce IAA (Duca et al., 2014; Patten and Glick, 1996). In plants, endogenously produced IAA serves as a phytohormone involved in the regulation of plant growth and development, including the root system. Exogenous application of IAA causes alterations in root system architecture that appear to depend on IAA concentration. For example,

low concentration of IAA generally stimulates primary root elongation, whereas high IAA levels may diminish primary root growth and stimulate the formation of lateral roots and root hairs ((Patten and Glick, 2002; Vacheron et al., 2013). The application of IAA-producing PGPR has been shown to produce similar root system responses, which have been linked to plant drought stress tolerance (Bresson et al., 2013; Marulanda et al., 2009; Ngumbi and Kloepper, 2016). Moreover, the specific role of IAA in mediating these phenotypes was demonstrated via comparison of growth promoting activity by auxin-producing PGPR and auxin-deficient mutants ((Patten and Glick, 2002; Vacheron et al., 2013). For example, canola seedlings treated with the auxin-producing PGPR *Pseudomonas putida* GR12-2 produced longer roots compared to seedlings treated with an auxin-deficient mutant or the untreated control. Cell-free supernatants of the wild type also enhanced the proliferation of adventitious roots on mung bean cuttings compared to supernatants of the mutant or the control (Patten and Glick, 2002; Vacheron et al., 2013). In contrast, bacterial production of IAA at high concentrations may have inhibitory on root growth and elongation, as demonstrated by the application of IAA overexpression derivatives (Sarwar and Kremer, 1995; Xie et al., 1996). In the present study, the alterations in root system architecture of both wheat and maize seedlings associated with the application of either strain are consistent with the hypothesis that bacterially-produced IAA may have contributed to these phenotypes, and this hypothesis merits further investigation.

Production of SA among rhizosphere-colonizing bacteria has been shown to be widespread and some strains can produce significant amounts when cultivated *in vitro.* For example, there are reported cases of *Pseudomonas fluorescens* biocontrol SA "super-producers" that can synthesize concentrations of SA up to 55µg per ml *in vitro* (Bakker et al., 2014). SA production may be

significantly increased under water stress, as observed for PGPR strains *Achromobacter xylosoxidans*, *B. pumilus* SF3, and *B. pumilus* SF4 (Forchetti et al., 2010). In plants, endogenously produced SA serves as a phytohormone involved in stress response. Although primarily studied for its involvement in activating systemic acquired resistance SAR in defense of biotic stresses, SA has also been shown to aid in abiotic stress tolerance, including drought (Khan et al., 2018; Wituszynska et al., 2013). Both phytohormones SA and abscisic acid (ABA) have been proposed to increase drought tolerance through the accumulation of induced reactive oxygen species (ROS) and induced signaling of stomatal closure (Ahmad, 2014; Daszkowska-Golec and Szarejko, 2013). By eliciting stomatal closure, these phytohormones can reduce transpirational water loss and allow for increasing water storage in the above ground tissue during drought conditions. It is therefore intriguing to speculate that bacterial production of SA may be involved in abiotic stress tolerance via its contribution to the endogenously produced plant SA pools and SA signaling pathways. However, despite the numerous examples of PGPR that produce SA and induce biotic or abiotic stress tolerance, there is very little evidence for the direct role of bacterially produced SA in these processes (Bakker et al., 2014). As Bakker et al. argue in a 2014 review of rhizobacterial salicylate production, although many root-inhabiting bacteria produce salicylic acid (SA) *in vitro*, in the rhizosphere they most likely excrete SA primarily as SA-based siderophores under iron limiting conditions or as an adaptation to high temperature conditions when other siderophore molecules are no longer functioning (Bakker et al., 2014). In contrast to the lack of effect on plants, bacterially produced SA has been shown to be involved in the regulation of key bacterial traits necessary for rhizosphere survival and thus may be important for regulating bacterial community dynamics under drought stress conditions (Bakker et al., 2014). The production of SA by both strains selected

for root colonization under drought stress conditions via our bioprospecting pipeline would seem to support this hypothesis.

In summary, the application of a high throughput bioprospecting pipeline to effectively screen PGPR for the capacity to rapidly mediate seedling drought stress symptoms identified two candidates: *Bacillus* sp. (12D6) and *Enterobacter* sp. (16i). Compared to untreated controls, both wheat and maize seedlings treated with either strain were significantly more vigorous following a 7-day water deficit and displayed alterations in root system architecture that likely facilitated the drought avoidance phenotype. Both strains produced IAA and SA *in vitro*. The ability of both strains to survive and rapidly protect both wheat and maize seedlings when applied at the onset of drought is a positive indicator of their potential for meditating seedling drought stress in cereal cropping systems and will be tested in future research.

CHAPTER III

HOST MEDIATED MICROBIOME ENGINEERING FOR DROUGHT TOLERANCE IN THE WHEAT RHIZOSPHERE

Synopsis

After six rounds of artificial selection using host-mediated microbiome engineering (HMME), a microbial community was selected that mediated a 5-day delay in the onset of drought symptoms in wheat seedlings. Seedlings grown in potting medium inoculated with the engineered rhizosphere from the 6^{th} round of HMME produced significantly more biomass and root system length, dry weight, and surface area than plants grown in medium similarly mixed with autoclaved inoculum (control plants). The effect on plant water stress tolerance conferred by the inoculum was transferable at subsequent 10- and 100-fold dilutions in fresh medium but was lost at 1000-fold dilution and was completely abolished by autoclaving, indicating the plant phenotype is mediated by microbial population dynamics. The results from next generation 16S rRNA amplicon sequencing of the rhizosphere microbiomes at rounds 0, 3, and 6 revealed taxonomic increases in proteobacteria at the phylum level and betaproteobacteria at the class level. There were significant decreases in alpha diversity in round 6, divergence in speciation with beta diversity between round 0 and 6, and changes in overall community composition. Functional metagenomic inferences based on Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSTt) suggested increases from round 0 to 6 in KEGG ortholog level 2 gene families associated with cell motility, cell signaling, and metabolism.

Introduction

Host-mediated microbiome engineering (HMME) is a cycle-dependent strategy that indirectly selects microbiomes based on host phenotype (Figure 8). For example, by directly selecting for increased seedling water stress tolerance, the host phenotype (e.g., delayed onset of seedling water deficit stress symptoms) is used to indirectly select for beneficial microbiome-host interactions over multiple generations using the same host germplasm. In this host-centric selection process, all microbiota are sub-selected at a community level, rather than on an individual basis (Swenson et al., 2000). This method allows microbiomes to change through both ecological (e.g., diversity, relative abundance) and evolutionary (e.g., extinction events, alterations in allele frequency, mutation, horizontal gene transfer) processes (Mueller and Sachs, 2015). Previous research demonstrated that HMME can indirectly select microbiomes for enhanced growth under altered soil pH by utilizing above ground biomass as a selection marker in *A. thaliana* (Swenson et al., 2000). Similarly, HMME was used to cultivate microbiomes capable of altering flowering onset and leaf biomass (Panke-Buisse et al., 2017; Panke-Buisse et al., 2014). In *Brachypodium distachyon*, results suggested HMME indirectly selected a rhizosphere microbiome that conferred salt-tolerance measured through the host phenotype (Mueller et al., 2016).

In the present study, I sought to use HMME to improve wheat seedling establishment under severe water stress associated with lack of rainfall, since seedling establishment is often the most vulnerable stage and may have large impacts on crop stand and yield (Pessarakli, 1994, 1999). The host phenotype used for screening was the delayed of onset of drought stress symptoms in wheat seedlings establishing under waster deficit conditions. The source of the original was obtained from the rhizospheres of perennial grasses collected from El Paso, TX, where the semi-arid environment provides a strong selective pressure for survival under nearly constant water deficit.

The rationale for choosing the starting material was that perennial grasses growing vigorously under pervasive water stress conditions were likely to foster a microbiome capable of mediating drought stress. In this HMME experiment, seeds of wheat cultivar TAM 111 (selected for enhanced drought tolerance), were planted into well-watered planting medium inoculated with the microbiome from the grassland rhizosphere soil. Water was then withheld until 90% of the seedlings showed symptoms of extreme drought stress (wilting to collapse). The plants displaying the least drought stress were selected and their rhizospheres (roots and planting medium) were then used as inoculum for subsequent selection cycles. Each of the subsequent selection cycles were similarly halted when 90% of the seedlings experienced collapse and again the rhizospheres of the best performing plants were used as inoculum. The cycling was terminated when there were no further improvements in time to wilting. Additional objectives of the study were to 1) determine whether changes in plant growth and development were associated with HMME-mediated improvements in water stress tolerance by comparing plants grown with non-autoclaved versus autoclaved inoculum from the final round of selection and 2) characterize changes in the taxonomic and functional diversity of the wheat seedling microbiomes during HMME selection rounds.

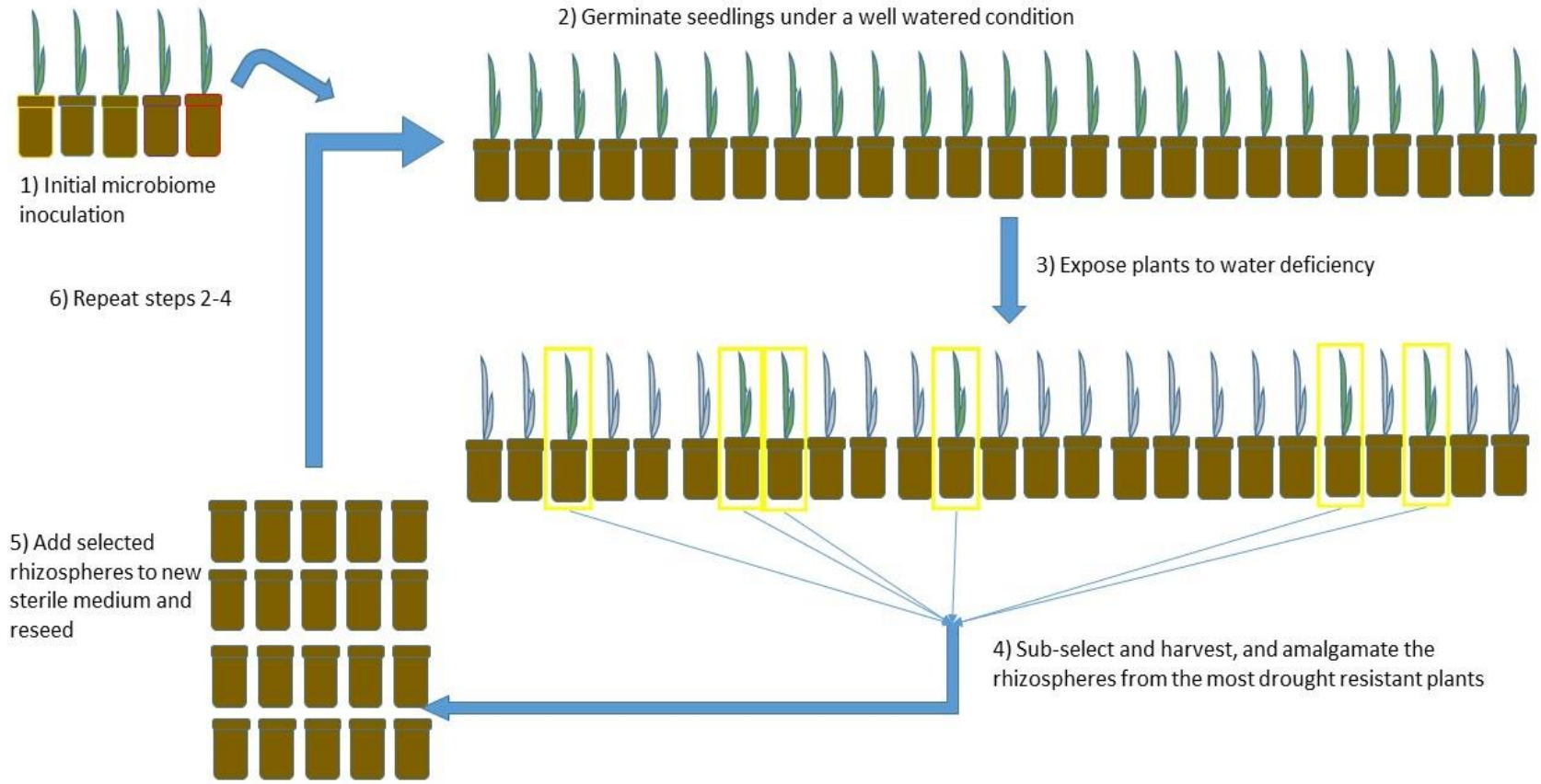

Figure 8 Concept of host mediated microbiome engineering**.** 1) An initial microbiome is inoculated. 2) Seeds are planted into well-watered conditions. 3) Emerging seedlings are then exposed to a drought stress by withholding watering. 4) When 90% of the plants display symptoms of water stress (wilting, leaf curling, etc.), the 5 best-performing plants are selected. Their rhizospheres (roots and planting medium) are amalgamated with autoclaved Metro-Mix 900 in a 1:10 ratio. The next round of selection is then initiated by planting seeds into the engineered planting medium.

Materials and Methods

Rhizobiome sampling

Twenty-five bermudagrass thatch core samples (10 cm diameter, 15 cm depth) were collected alongside medians, parks, roadsides, and ranches in the summer of 2016 in El Paso, Texas, USA. Intact core samples were immediately shipped upon removal under ambient temperatures to College Station, TX. Each sample core was then subdivided into 5 cm diameter cores, transferred to a round plastic pot (10 cm diameter, 8 cm height), filled-in with autoclaved potting mix (Metro-Mix 900, Sun Gro Horticulture, Agawam, MA), and grown in a greenhouse for 14 days. Grasses were exposed to three different levels of watering: non-stressed (watering up to the field capacity every other day), moderate stress (watering once a week), and severe stress (no watering). The onset of drought symptoms was monitored and recorded based on plant phenotype: leaf wilting, curling, tip burning, and plant lodging. The five cores containing plants for which drought symptoms were most delayed under both the moderate and severe watering regimes were used for the following microbiome engineering experiment.

Host mediated microbiome engineering

The entire root system from the selected grasses were separated from aboveground tissue and the root system and soil from the container were used as the inoculum for the initial HMME (Round 0). The inoculum was combined with autoclaved potting medium (Metro-Mix 900) at a 1:10 ratio (soil: potting mix by volume), watered to saturation, and mixed thoroughly in a sterile autoclave bag. A total of 50 pots (10 cm diameter, 8 cm depth) each filled with 400 ml (by volume) of the amalgamated medium. Wheat (*Triticum aestivum* subsp. *aestivum* cultivar TAM111) seeds were surface sterilized in 10% NaOCl for 10 min, followed by 10 subsequent

washes in sterile dH_2O. On day 0, each pot was sown with 5 seeds and covered lightly. Pots were incubated without any further watering in a growth chamber at 30ºC, using fluorescent bulbs emitting 300 μmol m^{-2} s^{-1}, 12 hr. :12 hr. light / dark cycle. When 90% (45 out of 50) of the plants displayed symptoms of severe water stress (wilting to collapse), the 5 pots containing the best-performing plants were selected. The rhizospheres (roots and planting medium) from the selected pots were then amalgamated with fresh, autoclaved Metro-Mix 900 in a 1:10 ratio. The amalgamated medium was again watered to saturation and mixed thoroughly. The next round of selection was initiated by planting seeds into the engineered planting medium. The artificial selection cycles continued to the point when the number of days delay in the onset of drought symptoms was no longer increasing (Figure 8).

Plant phenotype

To determine the effect of HMME on plant growth and development, germinated wheat seeds were planted into medium containing non-autoclaved or autoclaved inoculum (control) from the final round of selection (e.g. 1:10 ratio inoculum to autoclaved Metro-Mix 900). Pots were watered to field capacity, given no additional watering, and maintained under the aforementioned growth chamber conditions. After 10 days, plants were harvested, and roots were washed on a fine mesh sieve to remove debris. Whole plant fresh weight (biomass) was measured, and then roots were separated from above ground tissue and scanned using a flatbed scanner (EPSON, Perfection V-750). Intact root systems were transferred to a root positioning tray (20 cm × 30 cm) with sterile water (three root systems per tray) and carefully spread out to avoid root overlap. Root scans were analyzed using WinRHIZO Arabidopsis 2017a (Regent Instruments Inc., Quebec, Canada, 2000), generating estimates of total root length, root surface area, and number of root tips as previously

described (Arsenault et al., 1995; Himmelbauer et al., 2004). Dry weights were obtained after scanning and compared as previously described (Garnier, 1992), This experiment was repeated once.

Effect of dilution of HMME inoculum

Rhizosphere inoculum from the final round of HMME was serially diluted to 10^{-1}, 10^{-2} and 10^{-3} by amalgamation with Metro-mix 900; Metro-mix 900 receiving no HMME inoculum was used as the control. Treatments were wetted, mixed, and added to pots, and seeds were planted as described previously. The pots received no further water and were incubated under the aforementioned growth chamber conditions. Plant water stress symptoms were compared among treatments on day 10. To determine water loss over time, pots were weighed every 48 hrs. and the percentage water loss was calculated as the change over 48 hr. periods standardized to initial pot weight.

Statistical analyses

Treatments were arranged in a completely randomized block design. Differences among HMME inoculation treatments in plant growth and development traits and percent water loss were analyzed by analysis of variance (ANOVA) using SAS version 9.3 software (SAS Inc., Cary, NC). Pairwise comparisons between the treatments were analyzed using a protected Fisher's least significant difference (LSD) test. All scripts used in SAS analysis can be found in Appendix B.

DNA extraction and 16s rRNA sequencing

Genomic DNA from rhizosphere samples were extracted using the ZymoBIOMICS® DNA Miniprep Kit, SKU D4300 (Zymo Research). A control sample using the ZymoBIOMICS® Microbial Community Standard was also used as a positive control for detecting bias and background contamination. DNA samples were then sent to Novogene (Novogene Corporation, Inc.) for PCR amplification of the V4 region of 16S rRNA using primer set 515f (GTGCCAGCMGCCGCGGTAA) /806r (GGACTACHVGGGTWTCTAAT) primers followed by next generation sequencing (NGS) on an illumina HiSeq 4000 (Thompson et al., 2017). Sample prep, PCR, library prep, and NGS sequencing were done in accordance with Novogene protocols (Novogene Inc.) (Cock et al., 2010; Erlich et al., 2008; Hansen et al., 2010). Generated paired-end 250 bp raw reads and metadata were uploaded to NCBI (SRA: SRP158143, BioProject: PRJNA486342).

Bioinformatic processing and analysis

All of the analyses were conducted in the command line environment and executed on the Ada supercluster at the high-performance research computing center (HPRC) at Texas A&M. Barcodes were removed from the NGS raw reads via the bioinformatics software package Trimmomatic (Bolger et al., 2014) and imported into QIIME 2 Core 2018.6 (www.qiime2.org) using q2cli interface. Inside the QIIME2 environment, forward and reverse sequences were merged, and paired-end sequences were denoised, dereplicated, and filtered for chimeras using the Divisive Amplicon Denoising Algorithm (DADA2) plugin (Callahan et al., 2016). Following DADA2, the sequences were aligned using MAFFT, masked for highly variable sequences using qiime2 mask, and converted into a phylogenetic tree using the QIIME2 diversity plugin pipeline

for exploring community diversity. The diversity pipeline analyzed sequences for core-metrics results, ANOVA, alpha diversity, beta diversity, and constructed principle component of analysis (PCoA) plots using Bray-Curtis dissimilarity and weighted UniFrac distances. After diversity analysis, the sequences were feature classified into organizational taxonomic units (OTUs) using the 99% green genes 13_8 reference database for the 515f/806r, visualized using taxa bar plots, and collapsed into a table for each taxa level.

In order to incorporate the feature table into the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSTt) bioinformatic software package, sequences were reclassified using the qiime2 vsearch closed reference for PICRUSt plugin and the Greengenes 13_8 reference database with a 97% cutoff and exported to biom format. Using the bioinformatic software package PICRUSt 1.1.3 (Langille et al., 2013), OTUs were normalized by copy number. Following normalization, the functional metagenome was predicted by multiplying the OTU abundance by the predicted functional trait abundance using KEGG pathways. The resulting table was then categorized at three hierarchical levels, allowing the interpretation of multiple pathways (one-to-many relationships) to be counted. Files in biom format for each hierarchical level from the predicted functional metagenomes were then imported into the Statistical Analysis of Metagenomic Profiles (STAMP) software package for graphical visualization and statistical analysis of the predicted metagenomic profiles (Parks et al., 2014).

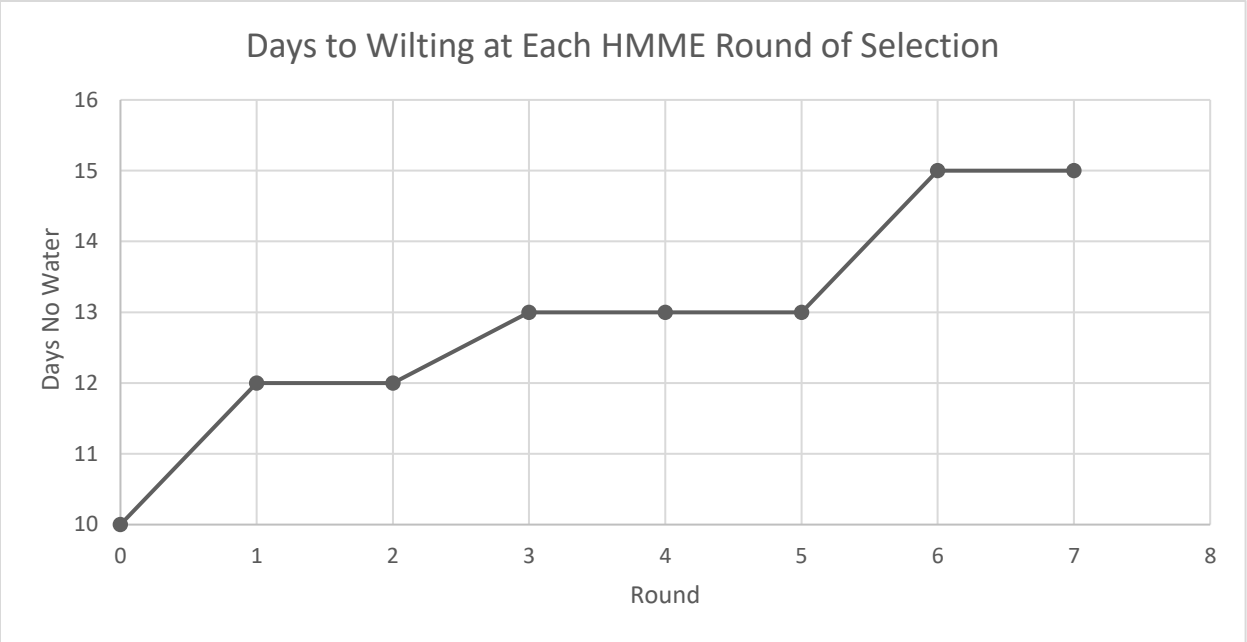

Figure 9 Effect of HMME rounds of selection on seedling drought tolerance. The number of days without water was determined as the day on which 90% of the seedlings displayed severe water stress symptoms (wilting to collapse.)

Figure 10 Image of HMME-mediated seedling phenotypes. Wheat seedlings growing in planting medium combined with either autoclaved (left) or non-autoclaved (right) rhizosphere inoculum from the final HMME round of selection after 10 days of withholding water.

Results

After six rounds of artificial selection using HMME, wheat seedlings exhibited a 5-day delay in the onset of drought stress symptoms compared to Round 0 (Figure 9). There were no differences in the number of days to the onset of drought symptom between Rounds 6 and 7. This water stress tolerance phenotype was abolished when plants were grown in medium amended with steam autoclaved HMME inoculum or potting mix containing no HMME inoculum, indicating that this phenotype was derived from a microbiome effect (Figures 10 and 12). Plants grown with HMME inoculum had significantly greater plant biomass, root dry weight, and total root length and surface area when compared to plants grown with autoclaved HMME inoculum (Figure 11, Table 3, Table 4). The effect of HMME inoculum on plant water stress tolerance was transferable at 10^{-1} and 10^{-2} dilutions but lost at the 10^{-3} dilution (Figure 12). On day 0, pot weights were significantly greater for pots with plants grown in the 10^{-1} dilution of inoculum as compared to pots with plants grown in the 10^{-3} dilution or control inoculum [10^{-1} = 293.59 g ± 2.11^{a}, 10^{-2} = 279.28 g ± 4.09^{ab}, 10^{-3} = 279.28 g ± 5.12^{b}, control = 280.10 g ± 2.11^{b}, $p \leq 0.05$]. These differences in initial pot weight confirm visual observations made following medium saturation and mixing that the HMME inoculum appeared to have enhanced soil aggregation and water holding capacity. Over the ten-day drying period, the percentage water loss from pots was generally less for treatments with more HMME inoculum. The difference in percent water loss was most apparent at days 4 and 6, where the average water loss for the HMME treatment was 22.39% and 37.23% compared to 36.90% and 44.91% for the control, respectively (Figure 13).

Table 3 Comparison of root system traits for plants grown with either non-autoclaved (HMME) or autoclaved HMME inoculum (control). Treatments were compared using an LSD Test (n=10). Letters indicate treatments that are statistically different ($P \leq 0.05$).

Treatment	Root Length (cm)	Biomass (mg)	Root Dry Weight (mg)	Root Surface Area (cm^2)	Tips
HMME	64.67^b	235.13^b	6.43^b	6.52^b	215.67
Control	47.97^a	158.60^a	4.62^a	5.02^a	171.17

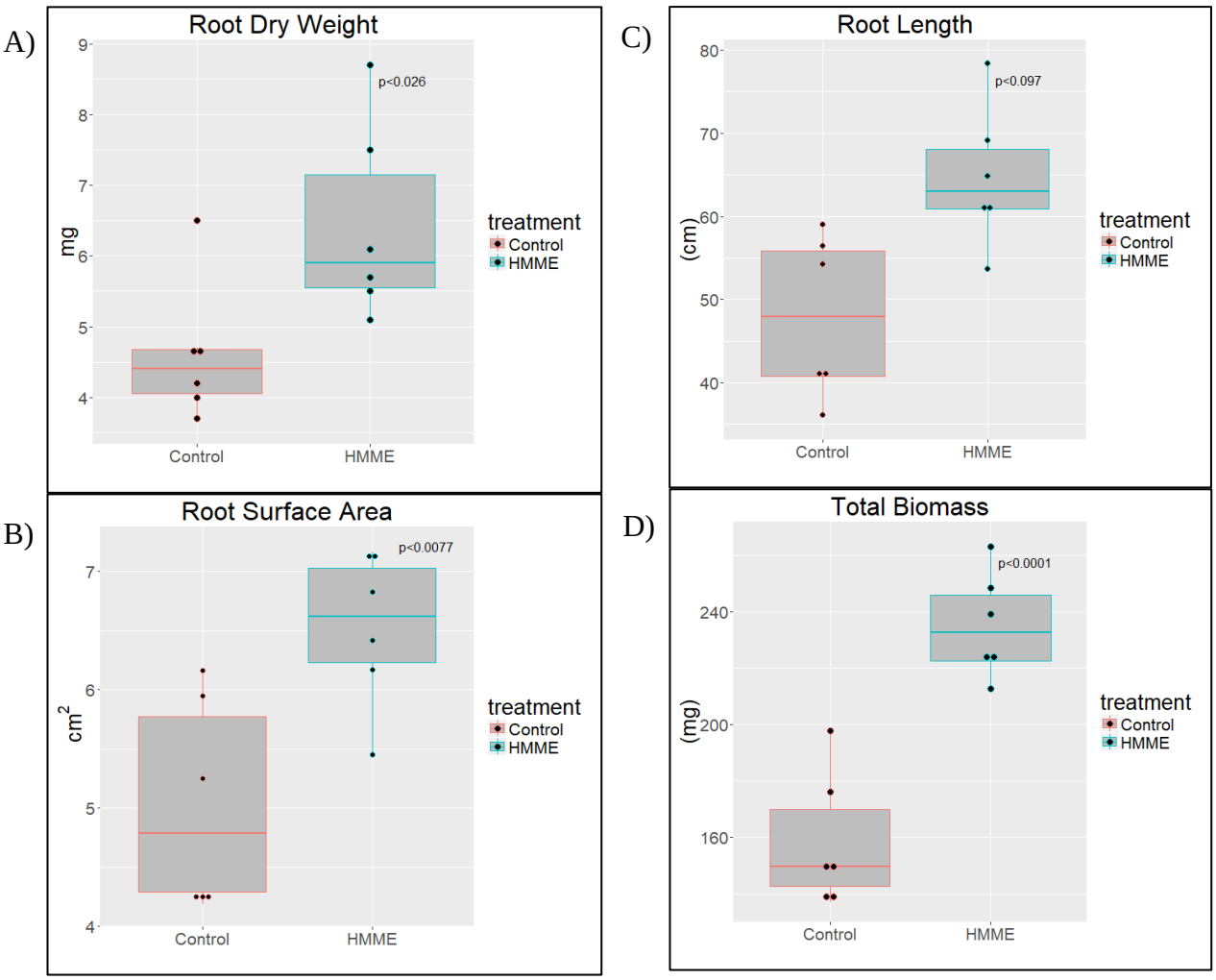

Figure 11 Comparison of root system traits for plants grown with either non-autoclaved (HMME) or autoclaved HMME inoculum (control). The box denotes the upper and lower quartiles (spans the interquartile range), the horizontal line denotes the median, and the vertical lines denote the highest and lowest observations.

Table 4 Analysis of variance (ANOVA) table for comparisons of seedling traits among HMME treatments (e.g. non-autoclaved vs. autoclaved HMME inoculum).

Dependent Variable	df	Mean squared	*F*	*P*
Root length	1	837.022407	10.15	0.0097
Total Biomass	1	17572.05333	39.04	< 0.0001
Root Dry Weight	1	9.90083333	6.82	0.0260
Root Surface Area	1	6.70657008	11.04	0.0077
Number of root tips	1	5940.75000	2.79	0.1258

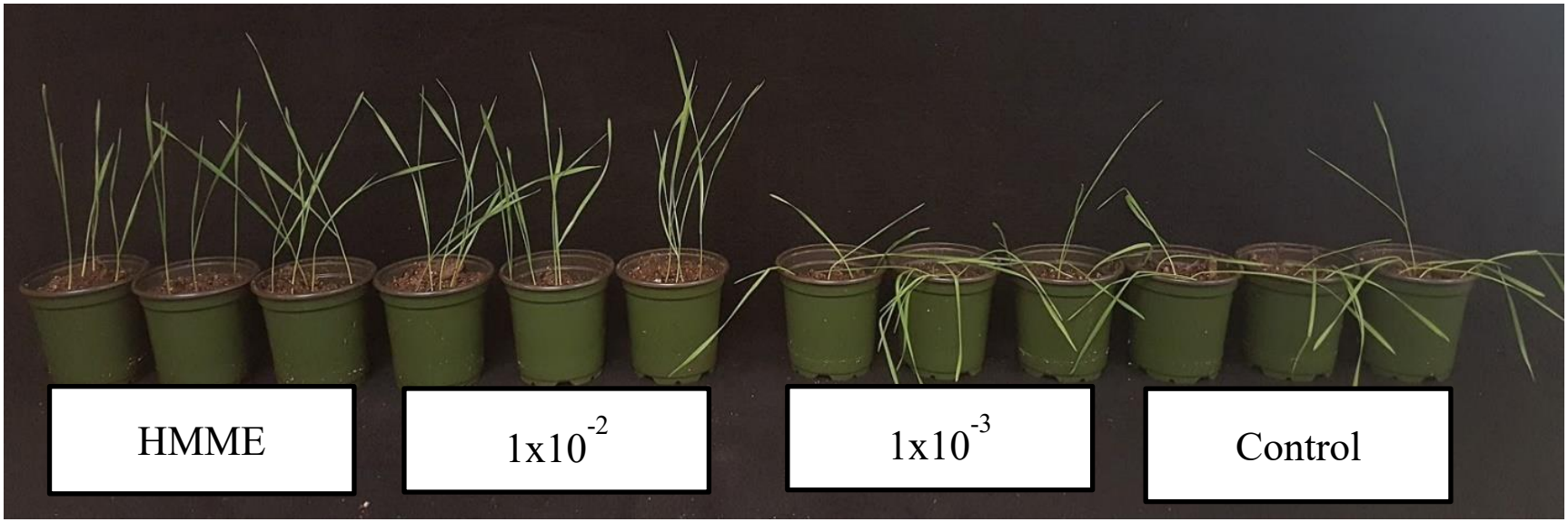

Figure 12 Transferability of the HMME mediated effect on plant water stress tolerance. Dilution of rhizosphere inoculum from HMME Round 6 demonstrates no loss of effectiveness in mediating the onset drought stress symptoms at day 10 for the $1x10^{-1}$ (HMME) and $1x10^{-2}$ dilution, but loss of efficacy at the $1x10^{-3}$ dilution, which displayed a similar phenotype to the treatment having no HMME inoculum (control).

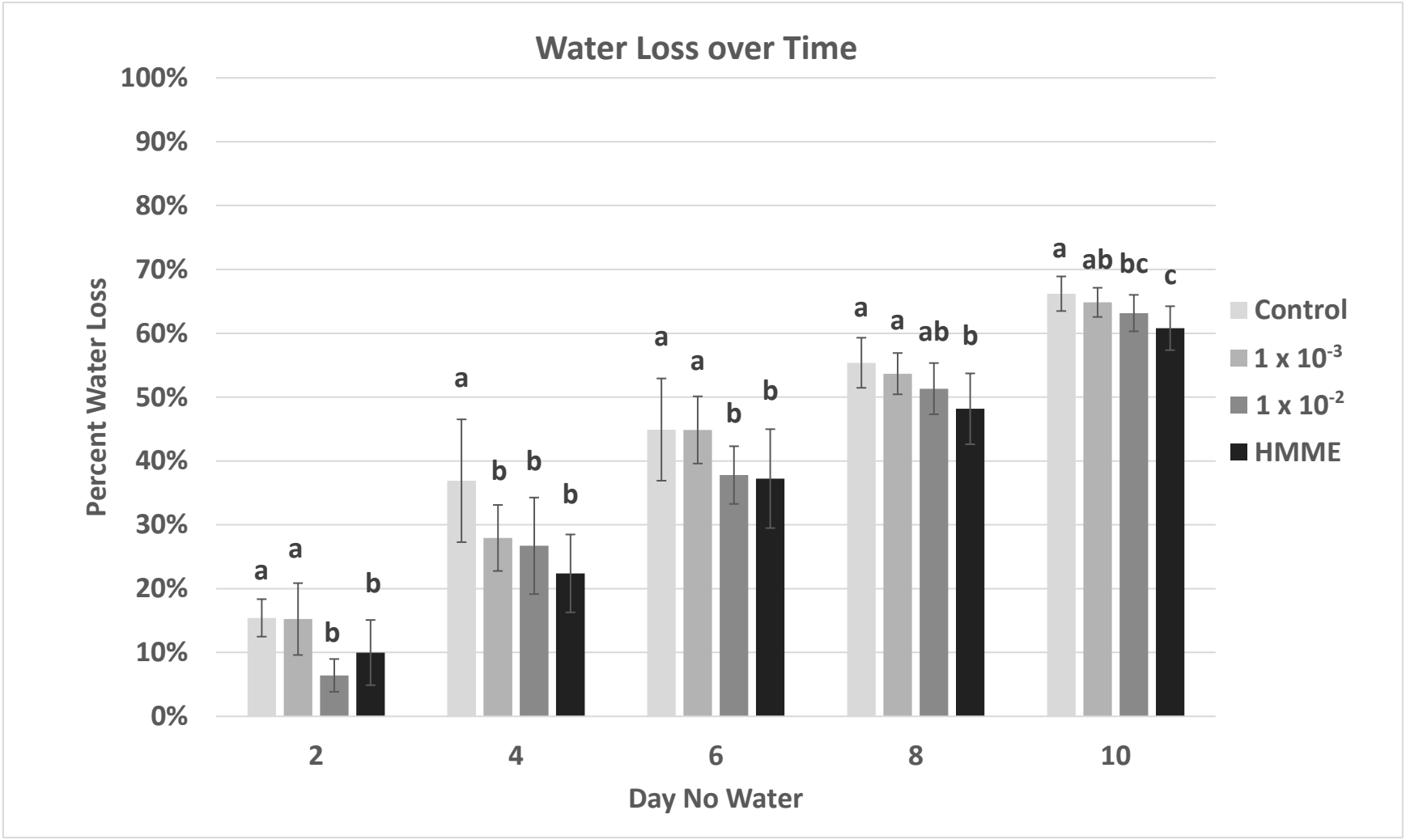

Figure 13 Transferability of the HMME mediated effect on water loss. Percentage of water loss calculated from the difference in pot weight every 48 hrs. relative to the starting pot weight. Treatments include pots growing seedlings inoculated with either a dilution series of rhizosphere inoculum from HMME Round 6: $1x10^{-1}$ (HMME), $1x10^{-2}$, $1x10^{-3}$, or no HMME inoculum (control). Standard error bars are shown, and significant differences are indicated.

Taxonomic analysis

Results from the 16S rRNA amplicon next generation sequencing revealed changes in relative microbial taxon abundance at both the phylum and class levels (Figure 14). The greatest change was the increase from 49.2% to 59.1% in the relative abundance of phyla proteobacteria when comparing round 0 (R0) to round 6 (R6) (Table 5). In contrast, when comparing R0 to R6, the relative abundance of actinobacteria (10.9% R0 to 6.2% R6) and acidobacteria (4.8% R0 to 2.4% R6) both decreased (Table 5). At the class level (Figure 14), there was over a threefold increase in the relative abundance of betaproteobacteria (7.3% R0 to 23.6% R6), a decrease in gammaproteobacteria (10.2% R0 to 8.5% R6), and marginal decreases in alphaproteobacteria (26.3% R0 to 20.8% R6) (Table 6).

Table 5 Phylum level analysis of variance (ANOVA) comparing HMME Rounds 0, 3, and 6.

Phylum	**Round 0**	**Round 3**	**Round 6**	***p*-value**
Proteobacteria	49.2	48.4	59.1	0.0670
Bacteroidetes	13.6	15.0	14.9	0.7130
Actinobacteria	10.9	9.0	6.2	0.1051
Firmicutes	7.4	6.0	5.4	0.4537
Verrucomicrobia	2.7	4.1	3.3	0.2004
Acidobacteria	4.8	4.2	2.4	0.0371
Chloroflexi	3.3	3.9	2.1	0.0743
Planctomycetes	1.8	2.8	2.0	0.3821
Cyanobacteria	1.7	1.7	1.5	0.9697
Gemmatimonadetes	1.5	1.3	0.7	0.0304

Table 6 Class level analysis of variance (ANOVA) comparing HMME Rounds 0, 3, and 6.

Class	**Round 0**	**Round 3**	**Round 6**	***p*-values**
Betaproteobacteria	7.3	7.6	23.6	0.008
Gammaproteobacteria	10.2	8.0	8.5	0.031
Deltaproteobacteria	5.1	8.0	5.5	0.055
Alphaproteobacteria	26.3	24.4	20.8	0.103

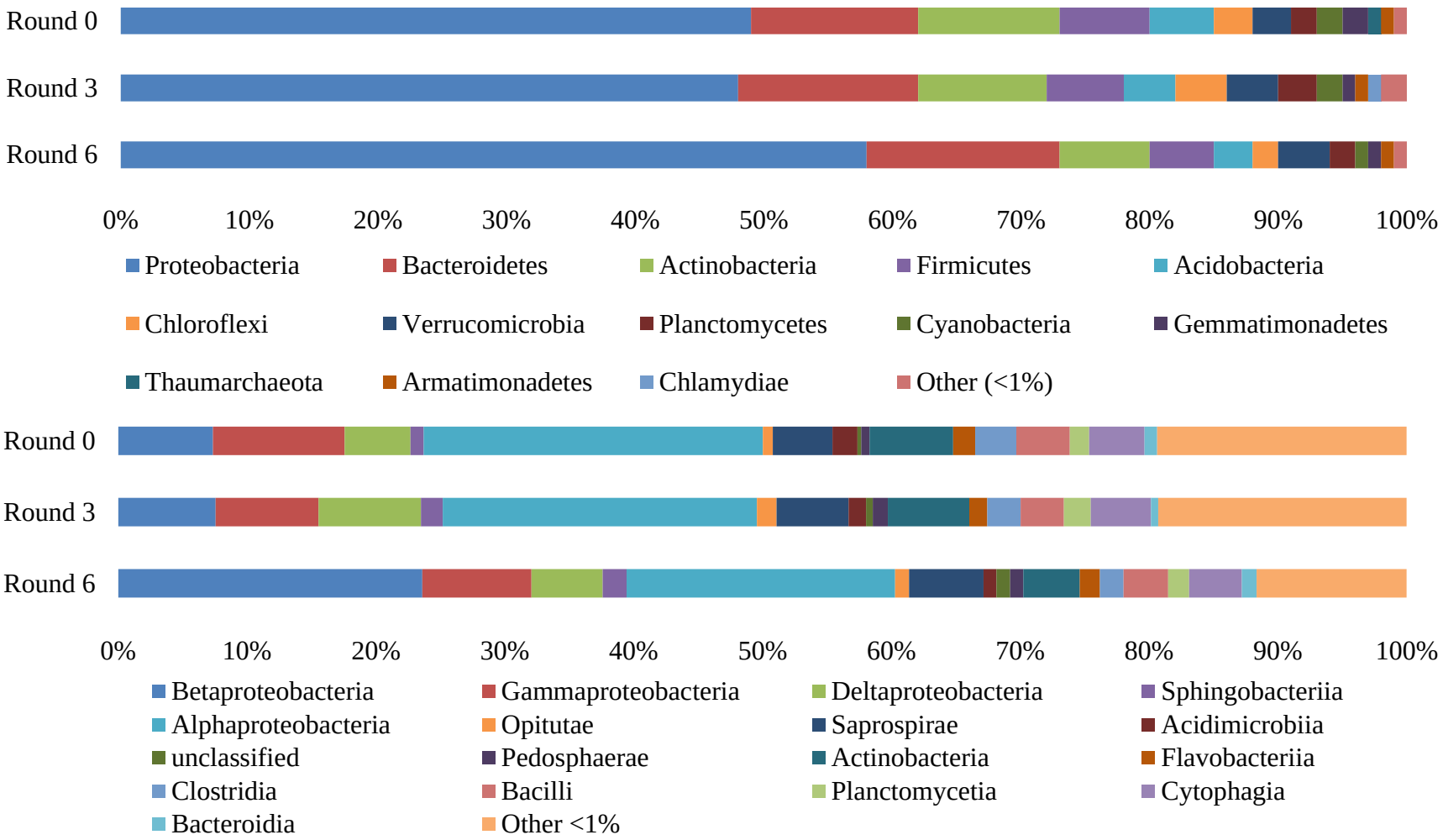

Figure 14 Taxonomic analysis. Stacked bar charts presenting relative abundance of each taxa at the phylum level (top) and class level (bottom). This comparison demonstrates host-mediated microbiome engineering resulted in taxonomic increases in Proteobacteria and Betaproteobacteria in the community rhizosphere when comparing round 0, 3 and 6. Taxa were referenced to the 99% OTU greengenes 13-8 database with a 1% abundance cutoff

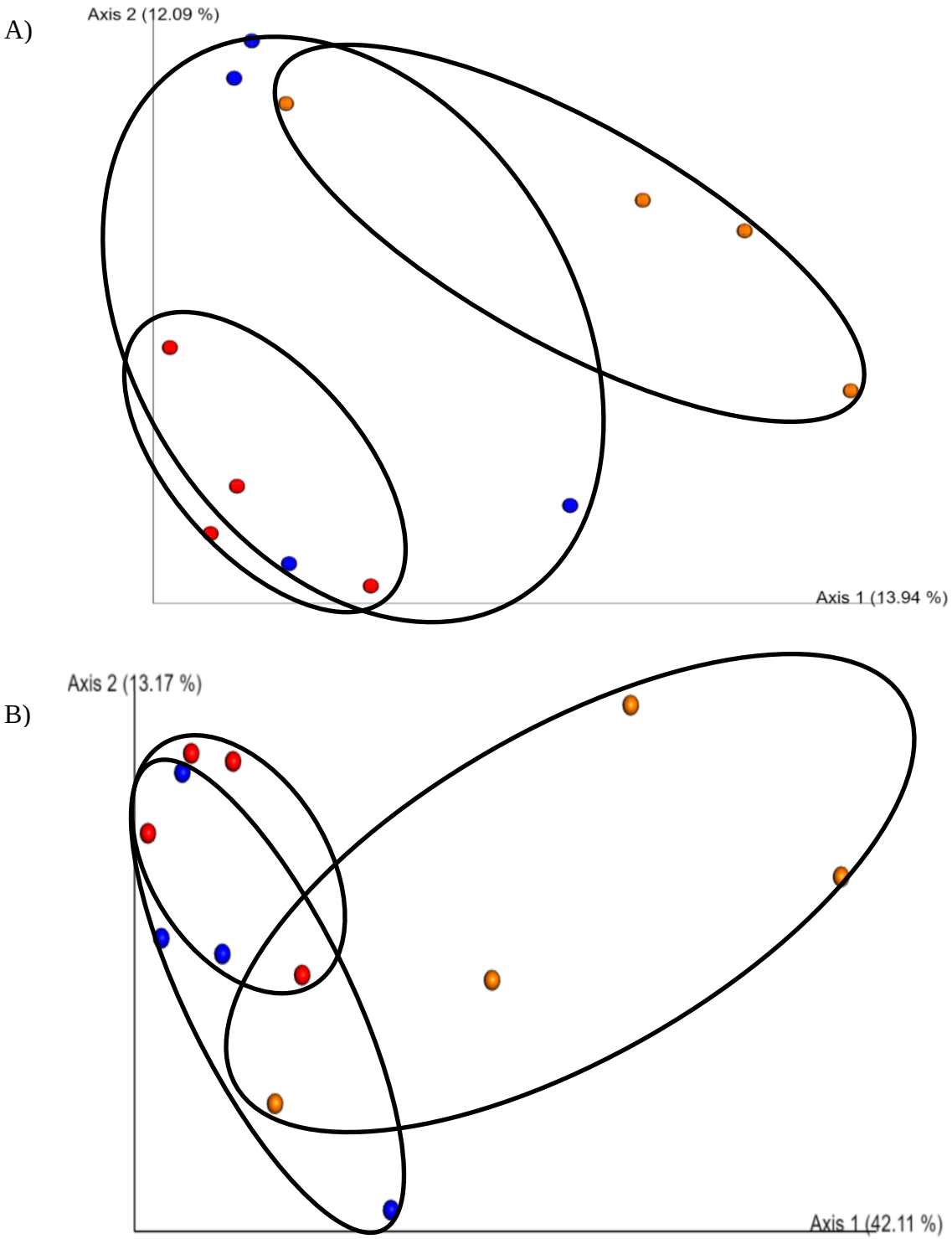

Figure 15 Beta-diversity. When comparing both a Bray Curtis dissimilarity index (A) and a Weighted UNIFRAC PCoA plot (B), successive generations show increasing divergence or dissimilarity from the original community extracted from R0 (red), with R3 (blue) transitioning between R0 and R6 (orange).

Phylogenetic diversity

Alpha diversity Simpson correlation using a Spearman's test statistic resulted in a significant decrease in alpha diversity when comparing R0 to R6 ($p = 0.048$). Beta diversity analysis using a PERMANOVA pseudo-*F* associated analysis with 999 permutations revealed a statistically significant difference when comparing R0 and R6 ($p = 0.029$), but not between R0 and R3 or R3 and R6 (Table 7). Beta diversity PCoA plots using a Bray Curtis dissimilarity index or a Weighted UNIFRAC index revealed dissimilarity in overall bacterial OTU composition between R0 and R6 with R3 transitioning between the two (Figure 15).

Table 7 Comparative analysis of R0, R3, and R6 using a PERMANOVA pseudo-F association analysis (permutations =999).

Group 1	Group 2	Sample size	Permutations	pseudo-F	*p*-value
R0	R3	8	999	1.165669	0.177
R0	R6	8	999	1.407776	0.029
R3	R6	8	999	1.200876	0.126

Predicted functional metagenome changes

Analysis of R0, R3, and R6 predicted functional metagenomic changes derived from PICRUSt level 2 KEGG orthologs suggested statistically significant increases in gene families involved in metabolism, signal transduction, cell processes and signaling, and cell motility (Table 8). Pairwise comparisons between R0 and R6 with 95% confidence revealed similar significant increases in the KEGG orthologs associated with metabolism, signal transduction, cell processes and signaling, and cell motility (Figure 16). The average nearest sequenced taxon index (NTSI), which reflect the relatedness to the reference genomes, were all less than 0.15 (> 85% similarity), indicating acceptable inference data quality (Langille et al., 2013).

Table 8 PICRUSt Orthologs. Predicted relative frequency of KEGG orthologs at level 2.

Function	R0	R3	R6	*p*-value
Metabolism	2.745 ± 0.498	2.837 ± 0.284	3.950 ± 0.205	0.025
Signal Transduction	3.391 ± 0.126	3.566 ± 0.080	3.900 ± 0.040	0.004
Cellular Processes and Signaling	2.556 ± 0.081	2.693 ± 0.032	2.975 ± 0.025	0.001
Cell Motility	2.083 ± 0.166	2.237 ± 0.131	2.863 ± 0.081	0.002

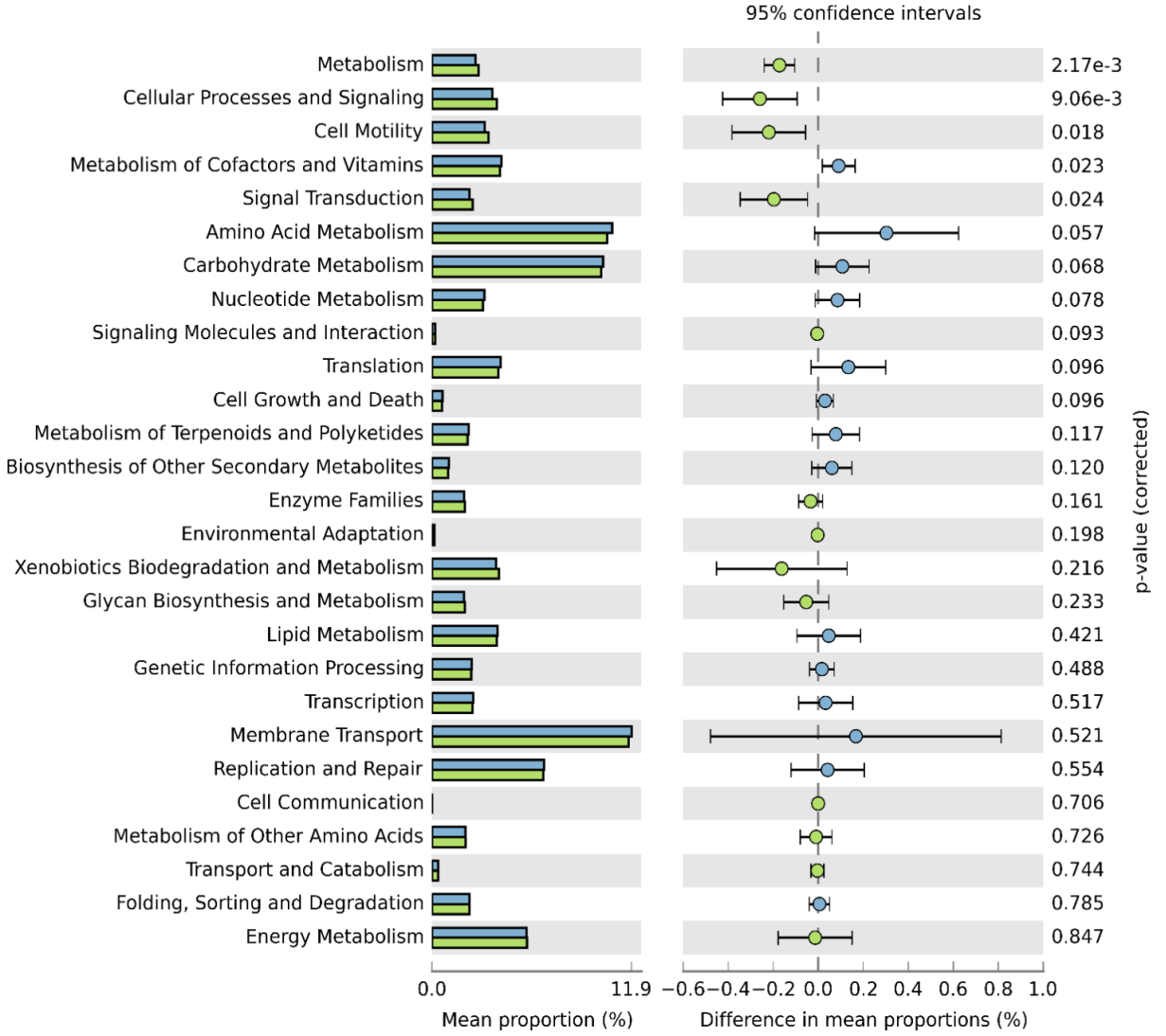

Figure 16 Predicted frequencies of KEGG orthologs. One-way comparison of R0 (blue) and R6 (green) predicted KEGG ortholog frequencies. Differences in mean proportions with 95% confidence intervals are given. Statistically significant increases in KEGG orthologs involved in cell motility, cell process and signaling, and signal transduction were found.

Discussion

This study provides the first example of utilizing HMME to indirectly select a rhizosphere microbiome that increased the seedling drought tolerance during water deficit conditions, by directly selecting for a delay in the onset of drought symptoms in newly established wheat seedlings. A steady increase in the ability of plants to survive water deficit occurred from the start of the experiment over the 6 rounds of HMME selection (drought stress symptom onset increased from 10 to 15 days—a gain of more than half a week before collapse). The improvements in drought tolerance stabilized after 6 rounds of HMME selection (and thus 6 rounds of 1:10 dilutions), and was transferable in subsequent 1:100 dilution, but lost if diluted further. The effect on seedling water stress tolerance was abolished by autoclaving the HMME inoculum. These results indicate that the change in plant adaptation to drought stress was a function of microbial population dynamics.

The increase in seedling drought tolerance with HMME was accompanied by changes in seedling growth and development. Seedlings grown with HMME inoculum were larger in size and had more extensive root system development than seedlings grown with autoclaved HMME inoculum. The observed alterations in plant growth and root system development are consistent with plant adaptation for maintaining plant productivity under drought (Comas et al., 2013). Previous research showed that increased root length and surface area contribute to increased soil exploration for available water (Barnawal et al., 2017; Naseem and Bano, 2014; Ngumbi and Kloepper, 2016; Vardharajula et al., 2011). It is unclear the extent to which the HMME microbiome may have contributed directly to plant growth and development via plant growth promoting activities and/or indirectly via modifications to the rhizosphere environment. For example, plant growth-promoting bacteria have been reported to contribute to plant growth and

root development via a number of mechanisms including suppression of seedling disease (although not a factor in this experiment) (Mendes et al., 2011), production of plant growth regulating compounds (Dimkpa et al., 2009; Dodd et al., 2010; Timmusk et al., 2014), assistance in nutrient uptake (Yang et al., 2009), and mediation of redox stress (Ngumbi and Kloepper, 2016). Additionally, previous research has demonstrated that extracellular polysaccharide production from beneficial microbiota provides significant indirect benefits to plant growth and development via improved soil structure and increased soil water retention (Chang et al., 2007; Naseem and Bano, 2014). These improvements coupled with higher respiration rates (and associated water release) by microbial communities selected for rapid growth and colonization of wheat seedling rhizospheres may lead to further improvements in water availability in the engineered microbiomes, therein enabling plants to avoid drought stress longer. Indeed, we observed (both visually and in terms of weight/volume) greater water retention in the HMME soil at the start and throughout the experiment.

Each round of HMME was associated with changes in taxonomic diversity and composition. As expected, dilution with HMME rounds of selection resulted in a reduction in alpha diversity (Yan et al. 2017). Comparison of beta diversity indicated potential host-mediated changes in rhizosphere populations with successive generations, showing increasing divergence or dissimilarity from the original community composition. Similar trends in species community structure associated with dilution were noted previously, although functional profiles of rhizosphere communities resulting from dilution overlapped more (Yan et al., 2017). Explaining this trend, the authors hypothesized that enrichment processes in the rhizosphere were more likely to select microbes with particular functionalities than taxonomies. In our study, taxonomic analysis based on next generation 16s rRNA amplicon sequencing of the rhizosphere microbiomes at

rounds 0, 3, and 6 revealed distinct changes in the microbial community, including increases in proteobacteria at the phylum level and betaproteobacteria at the class level. Functional metagenomic inferences based on PICRUSt suggested associated increases in KEGG ortholog level 2 gene families associated with cell motility, cell signaling, signal transduction, and metabolism from round 0 to 6. Although this inference is at too coarse of a scale to draw many conclusions, enrichment in motility and metabolism are consistent with selection for a microbiome that can colonize and proliferate quickly (e.g., within 10-15 days of germination) in the seedling rhizosphere as would be selected by our HMME protocol.

In summary, we showed that HMME can be used to enhance seedling drought tolerance at least under our experimental conditions. These results demonstrate the potential of engineered microbiomes to mediate changes in the rhizosphere environment, effectuating improved plant adaptation to water stress.

CHAPTER IV

CONCLUSIONS

The overarching goal for this research was to alleviate drought stress in grasses through the modification of the rhizosphere microbiota. The first objective was to conduct a bioprospecting screen of bermudagrass rhizospheres from a semi-arid environment in order to isolate plant growth promoting rhizobacteria that confer drought resistance to multiple varieties of grasses. Findings from the screen identified two isolates of rhizobacteria, *Bacillus* sp. (12D6) and *Enterobacter* sp. (16i) , that were capable of delaying the onset of drought stress and altering root system architecture for traits associated with drought tolerance, but with some degree of host specific efficacy. Results from the LC-MS based metabolomic profiling revealed that both strains produced IAA and SA *in vitro*. These findings support the hypothesis that PGPR can be isolated from a semi-arid environment rhizosphere and confer drought tolerance in multiple hosts.

I speculate that the mechanisms associated with the PGPR strains *Bacillus* sp. (12D6) and *Enterobacter* sp. (16i) mediated drought stress may include the following:

- Direct production or manipulation of signaling phytohormones like indole-3-acetic acid (IAA), which can induce growth and expansion of the root system
- Extracellular production of large chain polysaccharide that add humectant properties to the soil, therein improving overall soil structure

Future research regarding these specific mechanisms of these PGPR could include LC-MS metabolomic profiling *in planta* and exopolysaccharide production characterization assays. Future research regarding this experiment could also include screening these PGPR for synergistic effects, field validation and application trials, and a synthetic microbiome reconstruction experiment.

The second objective was to alter the rhizosphere microbiomes to confer an increase in the delay of onset of drought stress in wheat by using the host phenotype as a selection marker. Results from this experiment demonstrated a generational increase in rhizosphere mediated drought tolerance up to an addition 5 days. There were also statistically significant increases in root system architecture phenotypes associated with drought tolerance and an increase in water retention. Results from the next generation sequencing assay revealed changes in phylogeny, a decrease in diversity, and changes in the inferred functional metagenome. These findings from this experiment therefore support the hypothesis that host mediated microbiome engineering can be used to alter the root rhizosphere microbiome. I speculate the mechanisms associated with the observed mitigation of drought stress in the engineered rhizosphere microbiome may include the following:

- The production of signals that alter root system architecture for increased surface area exploration yielding increased water uptake
- The production of EPS that acts as a biofilm, adding humectant properties to the rhizosphere, which therein increase water retention and prevent root desiccation
- Ecological and evolutionary optimization of the microbiota that inhabit the rhizosphere to confer drought stress alleviation

Future research to test these observations could include the following:

- Mechanistic studies like metabolomic profiling of the plants under duress from water deficit
- Exopolysaccharide production characterization assays of the microbiomes from each generation
- Whole shotgun metagenomic sequencing for hologenome characterization and the observation of plasmid exchange, horizontal gene transfer, and changes in allelic frequency

In addition, future research regarding HMME could include the utilization of a no-template control (NTC) consisting of bulk soil that is transferred and diluted after the conclusion of each round and the addition of "low lines" consisting of a selection and propagation of the most drought susceptible soils for comparative analysis. Future research could also include the use of HMME for other biotic and abiotic stressors (e.g., NaCl tolerance, pathogen suppression, tolerance for heat/cold).

Overall, the significant outcomes from this research yielded a high throughput screen and the acquisition of two PGPR strains that can be directly used in the alleviation of seedling drought stress in grasses, and a better understanding of the microbiome profile associated with a drought tolerant wheat rhizosphere. Findings from both studies improve our understanding of the ecological and evolutionary implications of plant-microbe interactions in a water deficient environment, with potential outcomes that can be directly used for the alleviation of drought stress for applications in turfgrass and cereal crop production.

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APPENDIX A

BIOINFORMATIC PROCESSING SCRIPTS AND METADATA

Github_HMME.tree

```
.
└── HMME
    ├── 1_qiime_import.sh
    ├── 2_dada2.sh
    ├── 3_analysis.sh
    ├── 4_workflow.sh
    ├── 5_closed_ref_for_picrust.sh
    ├── hmme.org
    ├── HMMEQiitaPrep.txt
    ├── HMME_QIITA.txt
    ├── README.org
    ├── README.org~
    ├── sample-metadata.txt
    ├── test
    │   ├── 0_trimmomatic_pe.sh
    │   ├── 1_qiime_import.sh
    │   ├── 2_dada2.sh
    │   ├── 3_analysis.sh
    │   ├── 4_workflow.sh
    │   ├── 5_closed_ref_for_picrust.sh
    │   ├── 6_PICRUSt.sh
    │   ├── 6_PICRUSt.sh~
    │   ├── hmme.org
    │   ├── hmme.org~
    │   ├── reads
    │   │   ├── raw
    │   │   │   ├── R0c_1.fq.gz
    │   │   │   ├── R0c_2.fq.gz
    │   │   │   ├── R0d_1.fq.gz
    │   │   │   ├── R0d_2.fq.gz
    │   │   │   ├── R0g_1.fq.gz
    │   │   │   ├── R0g_2.fq.gz
    │   │   │   ├── R0h_1.fq.gz
    │   │   │   ├── R0h_2.fq.gz
    │   │   │   ├── R3a_1.fq.gz
    │   │   │   ├── R3a_2.fq.gz
    │   │   │   ├── R3d_1.fq.gz
    │   │   │   ├── R3d_2.fq.gz
    │   │   │   ├── R3e_1.fq.gz
    │   │   │   ├── R3e_2.fq.gz
    │   │   │   ├── R3h_1.fq.gz
    │   │   │   ├── R3h_2.fq.gz
    │   │   │   ├── R6a_1.fq.gz
    │   │   │   ├── R6a_2.fq.gz
    │   │   │   ├── R6c_1.fq.gz
    │   │   │   ├── R6c_2.fq.gz
    │   │   │   ├── R6d_1.fq.gz
    │   │   │   ├── R6d_2.fq.gz
```

```
│   │   │   ├── R6h_1.fq.gz
│   │   │   └── R6h_2.fq.gz
│   │   └── trimmed
│   │       ├── R0c_1_paired.fastq.gz
│   │       ├── R0c_1_single.fastq.gz
│   │       ├── R0c_2_paired.fastq.gz
│   │       ├── R0d_1_paired.fastq.gz
│   │       ├── R0d_1_single.fastq.gz
│   │       ├── R0d_2_paired.fastq.gz
│   │       ├── R0g_1_paired.fastq.gz
│   │       ├── R0g_1_single.fastq.gz
│   │       ├── R0g_2_paired.fastq.gz
│   │       ├── R0h_1_paired.fastq.gz
│   │       ├── R0h_1_single.fastq.gz
│   │       ├── R0h_2_paired.fastq.gz
│   │       ├── R3a_1_paired.fastq.gz
│   │       ├── R3a_1_single.fastq.gz
│   │       ├── R3a_2_paired.fastq.gz
│   │       ├── R3d_1_paired.fastq.gz
│   │       ├── R3d_1_single.fastq.gz
│   │       ├── R3d_2_paired.fastq.gz
│   │       ├── R3e_1_paired.fastq.gz
│   │       ├── R3e_1_single.fastq.gz
│   │       ├── R3e_2_paired.fastq.gz
│   │       ├── R3h_1_paired.fastq.gz
│   │       ├── R3h_1_single.fastq.gz
│   │       ├── R3h_2_paired.fastq.gz
│   │       ├── R6a_1_paired.fastq.gz
│   │       ├── R6a_1_single.fastq.gz
│   │       ├── R6a_2_paired.fastq.gz
│   │       ├── R6c_1_paired.fastq.gz
│   │       ├── R6c_1_single.fastq.gz
│   │       ├── R6c_2_paired.fastq.gz
│   │       ├── R6d_1_paired.fastq.gz
│   │       ├── R6d_1_single.fastq.gz
│   │       ├── R6d_2_paired.fastq.gz
│   │       ├── R6h_1_paired.fastq.gz
│   │       ├── R6h_1_single.fastq.gz
│   │       └── R6h_2_paired.fastq.gz
│   ├── rep-seqs.qza
│   ├── table.qza
│   └── trimmomatic_output.txt
└── trimmed_scripts
    ├── 0_trimmomatic_pe.sh
    ├── 1_qiime_import.sh
    ├── 2_dada2.sh
    ├── 3_analysis.sh
    ├── 4_workflow.sh
    └── 5_closed_ref_for_picrust.sh

6 directories, 90 files
```

sample-metadata.tsv

```
#BSUB -L /bin/bash              # uses the bash login shell to initialize the
job's execution environment.
#BSUB -J trimmomatic            # job name
#BSUB -n 2                      # assigns 2 cores for execution
#BSUB -R "span[ptile=8]"        # assigns 2 cores per node
#BSUB -R "rusage[mem=2500]"     # reserves 2500MB memory per core
#BSUB -M 2500                   # sets to 2500MB per process enforceable
memory limit. (M * n)
#BSUB -W 1:00                   # sets to 1 hour the job's runtime wall-clock
limit.
#BSUB -o stdout.%J              # directs the job's standard output to
stdout.jobid
#BSUB -e stderr.%J              # directs the job's standard error to
stderr.jobid

module load Trimmomatic/0.36-Java-1.8.0_92

################################################################################
###
# TODO Edit these variables as needed:
for number in 1 4 7
do
             threads=2                        # make sure this is <= your BSUB
-n value

pe1_1= ./reads/'R'$number'_1_paired.fastq'
pe1_2= ./reads/'R'$number'_2_paired.fastq'

prefix='R'$number'_original'
min_length=150
quality_format="-phred33"       # -phred33, -phred64    # see
https://en.wikipedia.org/wiki/FASTQ_format#Encoding

adapter_file='TruSeq3-PE.fa'
# available adapter files:
#   Nextera:      NexteraPE-PE.fa
#   GAII:         TruSeq2-PE.fa, TruSeq2-SE.fa
#   HiSeq,MiSeq:  TruSeq3-PE-2.fa, TruSeq3-PE.fa, TruSeq3-SE.fa

################################################################################
###
#
java -jar $EBROOTTRIMMOMATIC/trimmomatic-0.36.jar \
PE -threads $threads $quality_format $pe1_1 $pe1_2 \
preefix_pe1_trimmo.fastq.gz  \
preefix_pe2_trimmo.fastq.gz  \
ILLUMINACLIP:$EBROOTTRIMMOMATIC/adapters/$adapter_file:2:30:10 \
MINLEN:$min_length

          done

<<CITATION
    - Acknowledge TAMU HPRC: https://hprc.tamu.edu/research/citations.html
```

- Trimmomatic:
Anthony M. Bolger1,2, Marc Lohse1 and Bjoern Usadel. Trimmomatic: A flexible trimmer for Illumina Sequence Data.
Bioinformatics. 2014 Aug 1;30(15):2114-20. doi: 10.1093/bioinformatics/btu170.
CITATION

sample-metadata-stamp.tsv

```
Sample Id       BarcodeSequence LinkerPrimerSequence    SampleSite      Year
        Month   Day     Subject Round   Start Date      Experiment Stop Date
        DaysNo Water    Description
R0h     CCAGTTCA        GTGCCAGCMGCCGCGGTAA     Rhizosphere     2017    5       5
        Round-1 1       13-Oct-16       20-Oct-16       10      Round 1
Subsample A
R0c     ACGTATCA        GTGCCAGCMGCCGCGGTAA     Rhizosphere     2017    5       5
        Round-1 1       13-Oct-16       20-Oct-16       10      Round 1
Subsample B
R0d     ACGTATCA        GTGCCAGCMGCCGCGGTAA     Rhizosphere     2017    5       5
        Round-1 1       13-Oct-16       20-Oct-16       10      Round 1
Subsample C
R0g     ACGTATCA        GTGCCAGCMGCCGCGGTAA     Rhizosphere     2017    5       5
        Round-1 1       13-Oct-16       20-Oct-16       10      Round 1
Subsample D
R3h     CCAGTTCA        GTGCCAGCMGCCGCGGTAA     Rhizosphere     2017    5       5
        Round-4 4       5-Jan-17        19-Jan-17       13      Round 2
Subsample A
R3a     ACGTATCA        GTGCCAGCMGCCGCGGTAA     Rhizosphere     2017    5       5
        Round-4 4       5-Jan-17        19-Jan-17       13      Round 2
Subsample B
R3d     ACGTATCA        GTGCCAGCMGCCGCGGTAA     Rhizosphere     2017    5       5
        Round-4 4       5-Jan-17        19-Jan-17       13      Round 2
Subsample C
R3e     ACGTATCA        GTGCCAGCMGCCGCGGTAA     Rhizosphere     2017    5       5
        Round-4 4       5-Jan-17        19-Jan-17       13      Round 2
Subsample D
R6h     CCAGTTCA        GTGCCAGCMGCCGCGGTAA     Rhizosphere     2017    5       5
        Round-7 7       15-Feb-17       3-Mar-17        15      Round 3
Subsample A
R6a     ACGTATCA        GTGCCAGCMGCCGCGGTAA     Rhizosphere     2017    5       5
        Round-7 7       15-Feb-17       3-Mar-17        15      Round 3
Subsample B
R6c     ACGTATCA        GTGCCAGCMGCCGCGGTAA     Rhizosphere     2017    5       5
        Round-7 7       15-Feb-17       3-Mar-17        15      Round 3
Subsample C
R6d     ACGTATCA        GTGCCAGCMGCCGCGGTAA     Rhizosphere     2017    5       5
        Round-7 7       15-Feb-17       3-Mar-17        15      Round 3
Subsample D
```

sample-metadata-qiita.tsv

```
sample_name     TITLE   ANONYMIZED_NAME scientific_name TAXON_ID
        DESCRIPTION     sample_type     geo_loc_name    ENV_BIOME
        ENV_FEATURE     ENV_MATERIAL    ENV_package     LATITUDE        LONGITUDE
        COLLECTION_TIMESTAMP    dna_extracted   physical_specimen_location
        physical_specimen_remaining     host_age        host_subject_id
        host_taxid      host_scientific_name    host_common_name
        host_life_stage sex     host_height     height_units    host_weight
        weight_units    host_body_mass_index    host_body_habitat
        host_body_site  host_body_product       altitude        empo_1  empo_2
        empo_3  BarcodeSequence LinkerPrimerSequence    SampleSite      Year
        Month   Day     Subject Round   start_date      stop_date
        days_no_water
R0h     Host mediated microbiome engineering drought tolerance in the wheat
rhizosphere     R0h     rhizosphere metagenome 939928  root and root adherent
soil    rhizome USA:TX:El Paso  desert scrubland        plant-associated habitat
        organic material/soil  organic material/soil  31.75   -106.4
        10/13/2016      TRUE    Texas A&M       TRUE    seedling        root 1h
        4565    Triticum aestivum subsp. aestivum      plants  seedling
        Not applicable Not provided    Not provided    Not provided    Not
provided        Not applicable Not applicable Not applicable Not applicable
        Not applicable Host-asssociated        Plant-associated        Plant
corpus  CCAGTTCA        GTGCCAGCMGCCGCGGTAA    Rhizosphere     2017    5       5
        Round-1 1       13-Oct-16       20-Oct-16       10
R0c     Host mediated microbiome engineering drought tolerance in the wheat
rhizosphere     R0c     rhizosphere metagenome 939928  root and root adherent
soil    rhizome USA:TX:El Paso  desert scrubland        plant-associated habitat
        organic material/soil  organic material/soil  31.75   -106.4
        10/13/2016      TRUE    Texas A&M       TRUE    seedling        root 1c
        4565    Triticum aestivum subsp. aestivum      plants  seedling
        Not applicable Not provided    Not provided    Not provided    Not
provided        Not applicable Not applicable Not applicable Not applicable
        Not applicable Host-asssociated        Plant-associated        Plant
corpus  ACGTATCA        GTGCCAGCMGCCGCGGTAA    Rhizosphere     2017    5       5
        Round-1 1       13-Oct-16       20-Oct-16       10
R0d     Host mediated microbiome engineering drought tolerance in the wheat
rhizosphere     R0d     rhizosphere metagenome 939928  root and root adherent
soil    rhizome USA:TX:El Paso  desert scrubland        plant-associated habitat
        organic material/soil  plant-associated        31.75   -106.4
        10/13/2016      TRUE    Texas A&M       TRUE    seedling        root 1d
        4565    Triticum aestivum subsp. aestivum      plants  seedling
        Not applicable Not provided    Not provided    Not provided    Not
provided        Not applicable Not applicable Not applicable Not applicable
        Not applicable Host-asssociated        Plant-associated        Plant
corpus  ACGTATCA        GTGCCAGCMGCCGCGGTAA    Rhizosphere     2017    5       5
        Round-1 1       13-Oct-16       20-Oct-16       10
R0g     Host mediated microbiome engineering drought tolerance in the wheat
rhizosphere     R0g     rhizosphere metagenome 939928  root and root adherent
soil    rhizome USA:TX:El Paso  desert scrubland        plant-associated habitat
        organic material/soil  plant-associated        31.75   -106.4
        10/13/2016      TRUE    Texas A&M       TRUE    seedling        root 1g
        4565    Triticum aestivum subsp. aestivum      plants  seedling
        Not applicable Not provided    Not provided    Not provided    Not
provided        Not applicable Not applicable Not applicable Not applicable
```

Not applicable Host-asssociated Plant-associated Plant corpus ACGTATCA GTGCCAGCMGCCGCGGTAA Rhizosphere 2017 5 5 Round-1 1 13-Oct-16 20-Oct-16 10
R3h Host mediated microbiome engineering drought tolerance in the wheat rhizosphere R3h rhizosphere metagenome 939928 root and root adherent soil rhizome USA:TX:El Paso desert scrubland plant-associated habitat organic material/soil plant-associated 31.75 -106.4 1/5/2017 TRUE Texas A&M TRUE seedling root 4h 4565 Triticum aestivum subsp. aestivum plants seedling Not applicable Not provided Not provided Not provided Not provided Not applicable Not applicable Not applicable Not applicable Not applicable Host-asssociated Plant-associated Plant corpus CCAGTTCA GTGCCAGCMGCCGCGGTAA Rhizosphere 2017 5 5 Round-4 4 5-Jan-17 19-Jan-17 13
R3a Host mediated microbiome engineering drought tolerance in the wheat rhizosphere R3a rhizosphere metagenome 939928 root and root adherent soil rhizome USA:TX:El Paso desert scrubland plant-associated habitat organic material/soil plant-associated 31.75 -106.4 1/5/2017 TRUE Texas A&M TRUE seedling root 4a 4565 Triticum aestivum subsp. aestivum plants seedling Not applicable Not provided Not provided Not provided Not provided Not applicable Not applicable Not applicable Not applicable Not applicable Host-asssociated Plant-associated Plant corpus ACGTATCA GTGCCAGCMGCCGCGGTAA Rhizosphere 2017 5 5 Round-4 4 5-Jan-17 19-Jan-17 13
R3d Host mediated microbiome engineering drought tolerance in the wheat rhizosphere R3d rhizosphere metagenome 939928 root and root adherent soil rhizome USA:TX:El Paso desert scrubland plant-associated habitat organic material/soil plant-associated 31.75 -106.4 1/5/2017 TRUE Texas A&M TRUE seedling root 4d 4565 Triticum aestivum subsp. aestivum plants seedling Not applicable Not provided Not provided Not provided Not provided Not applicable Not applicable Not applicable Not applicable Not applicable Host-asssociated Plant-associated Plant corpus ACGTATCA GTGCCAGCMGCCGCGGTAA Rhizosphere 2017 5 5 Round-4 4 5-Jan-17 19-Jan-17 13
R3e Host mediated microbiome engineering drought tolerance in the wheat rhizosphere R3e rhizosphere metagenome 939928 root and root adherent soil rhizome USA:TX:El Paso desert scrubland plant-associated habitat organic material/soil plant-associated 31.75 -106.4 1/5/2017 TRUE Texas A&M TRUE seedling root 4d 4565 Triticum aestivum subsp. aestivum plants seedling Not applicable Not provided Not provided Not provided Not provided Not applicable Not applicable Not applicable Not applicable Not applicable Host-asssociated Plant-associated Plant corpus ACGTATCA GTGCCAGCMGCCGCGGTAA Rhizosphere 2017 5 5 Round-4 4 5-Jan-17 19-Jan-17 13
R6h Host mediated microbiome engineering drought tolerance in the wheat rhizosphere R6h rhizosphere metagenome 939928 root and root adherent soil rhizome USA:TX:El Paso desert scrubland plant-associated habitat organic material/soil plant-associated 31.75 -106.4 2/15/2017 TRUE Texas A&M TRUE seedling root 6h 4565 Triticum aestivum subsp. aestivum plants seedling Not applicable Not provided Not provided Not provided Not provided Not applicable Not applicable Not applicable Not applicable Not applicable Host-

asssociated Plant-associated Plant corpus CCAGTTCA
GTGCCAGCMGCCGCGGTAA Rhizosphere 2017 5 5 Round-7 7
15-Feb-17 3-Mar-17 15
R6a Host mediated microbiome engineering drought tolerance in the wheat
rhizosphere R6a rhizosphere metagenome 939928 root and root adherent
soil rhizome USA:TX:El Paso desert scrubland plant-associated habitat
organic material/soil plant-associated 31.75 -106.4 2/15/2017
TRUE Texas A&M TRUE seedling root 6a 4565 Triticum
aestivum subsp. aestivum plants seedling Not applicable Not
provided Not provided Not provided Not provided Not applicable
Not applicable Not applicable Not applicable Not applicable Host-
asssociated Plant-associated Plant corpus ACGTATCA
GTGCCAGCMGCCGCGGTAA Rhizosphere 2017 5 5 Round-7 7
15-Feb-17 3-Mar-17 15
R6c Host mediated microbiome engineering drought tolerance in the wheat
rhizosphere R6c rhizosphere metagenome 939928 root and root adherent
soil rhizome USA:TX:El Paso desert scrubland plant-associated habitat
organic material/soil plant-associated 31.75 -106.4 2/15/2017
TRUE Texas A&M TRUE seedling root 6c 4565 Triticum
aestivum subsp. aestivum plants seedling Not applicable Not
provided Not provided Not provided Not provided Not applicable
Not applicable Not applicable Not applicable Not applicable Host-
asssociated Plant-associated Plant corpus ACGTATCA
GTGCCAGCMGCCGCGGTAA Rhizosphere 2017 5 5 Round-7 7
15-Feb-17 3-Mar-17 15
R6d Host mediated microbiome engineering drought tolerance in the wheat
rhizosphere R6d rhizosphere metagenome 939928 root and root adherent
soil rhizome USA:TX:El Paso desert scrubland plant-associated habitat
organic material/soil plant-associated 31.75 -106.4 2/15/2017
TRUE Texas A&M TRUE seedling root 6d 4565 Triticum
aestivum subsp. aestivum plants seedling Not applicable Not
provided Not provided Not provided Not provided Not applicable
Not applicable Not applicable Not applicable Not applicable Host-
asssociated Plant-associated Plant corpus ACGTATCA
GTGCCAGCMGCCGCGGTAA Rhizosphere 2017 5 5 Round-7 7
15-Feb-17 3-Mar-17 15

0_trimmomatic_pe.sh

```
#BSUB -L /bin/bash              # uses the bash login shell to initialize the
job's execution environment.
#BSUB -J trimmomatic            # job name
#BSUB -n 2                      # assigns 2 cores for execution
#BSUB -R "span[ptile=8]"        # assigns 2 cores per node
#BSUB -R "rusage[mem=2500]"     # reserves 2500MB memory per core
#BSUB -M 2500                   # sets to 2500MB per process enforceable
memory limit. (M * n)
#BSUB -W 1:00                   # sets to 1 hour the job's runtime wall-clock
limit.
#BSUB -o stdout.%J              # directs the job's standard output to
stdout.jobid
#BSUB -e stderr.%J              # directs the job's standard error to
stderr.jobid

module load Trimmomatic/0.36-Java-1.8.0_92

################################################################################
###
# TODO Edit these variables as needed:
for number in 1 4 7
do
             threads=2                        # make sure this is <= your BSUB
-n value

pe1_1= ./reads/'R'$number'_1_paired.fastq'
pe1_2= ./reads/'R'$number'_2_paired.fastq'

preefix='R'$number'_original'
min_length=150
quality_format="-phred33"       # -phred33, -phred64    # see
https://en.wikipedia.org/wiki/FASTQ_format#Encoding

adapter_file='TruSeq3-PE.fa'
# available adapter files:
#   Nextera:      NexteraPE-PE.fa
#   GAII:         TruSeq2-PE.fa, TruSeq2-SE.fa
#   HiSeq,MiSeq:  TruSeq3-PE-2.fa, TruSeq3-PE.fa, TruSeq3-SE.fa

################################################################################
###
#
java -jar $EBROOTTRIMMOMATIC/trimmomatic-0.36.jar \
PE -threads $threads $quality_format $pe1_1 $pe1_2 \
p_pe1_trimmo.fastq.gz  \
p_pe2_trimmo.fastq.gz  \
ILLUMINACLIP:$EBROOTTRIMMOMATIC/adapters/$adapter_file:2:30:10 \
MINLEN:$min_length

          done

<<CITATION
    - Acknowledge TAMU HPRC: https://hprc.tamu.edu/research/citations.html
```

- Trimmomatic:
Anthony M. Bolger1,2, Marc Lohse1 and Bjoern Usadel. Trimmomatic: A flexible trimmer for Illumina Sequence Data.
Bioinformatics. 2014 Aug 1;30(15):2114-20. doi: 10.1093/bioinformatics/btu170.
CITATION

1_qiime_import.sh

```
##NECESSARY JOB SPECIFICATIONS
#BSUB -J jochum_qiime         #Set the job name to "ExampleJob3"
#BSUB -L /bin/bash           #Uses the bash login shell to initialize the
job's execution environment.
#BSUB -W 24:00               #Set the wall clock limit to 24hr
#BSUB -n 40                  #Request 40 cores
#BSUB -R "span[ptile=20]"    #Request 20 cores per node.
#BSUB -R "rusage[mem=12000]"  #Request 2560MB per process (CPU) for the job
#BSUB -M 12000                #Set the per process enforceable memory limit
to 2560MB.
#BSUB -o qout.%J      #Send stdout and stderr to "Example3Out.[jobID]"
#BSUB -e qerr.%J
#First Executable Line

module load Anaconda/3-5.0.0.1
source activate qiime2-2018.2
qiime tools import \
  --type 'SampleData[PairedEndSequencesWithQuality]' \
  --input-path hmme.org \
  --output-path demux.qza \
  --source-format PairedEndFastqManifestPhred33
```

2_dada2.sh

```
#BSUB -J whole_jochum_qiime2         #Set the job name to "ExampleJob3"
#BSUB -L /bin/bash                 #Uses the bash login shell to initialize
the job's execution environment.
#BSUB -W 24:00                      #Set the wall clock limit to 24hr
#BSUB -n 20                        #Request 40 cores
#BSUB -R "span[ptile=20]"          #Request 20 cores per node.
#BSUB -R "rusage[mem=2560]"        #Request 2560MB per process (CPU) for the
job
#BSUB -M 2560                      #Set the per process enforceable memory
limit to 2560MB.
#BSUB -o
/scratch/group/ykjolab/jochumHMMEqiime/HMME_QIIME/artifacts/18June18/whole_qi
imeout.%J
#BSUB -e
/scratch/group/ykjolab/jochumHMMEqiime/HMME_QIIME/artifacts/18June18/whole_qi
imerr.%J

#First Executable Line
module load Anaconda/3-5.0.0.1
source activate qiime2-2018.2
module load R_tamu/3.4.2-intel-2017A-Python-2.7.12-default-mt
#========= DADA2 =========
qiime dada2 denoise-paired \
  --i-demultiplexed-seqs demux.qza \
  --p-trim-left-f 0 \
  --p-trim-left-r 0 \
  --p-trunc-len-f 244 \
  --p-trunc-len-r 244 \
  --o-table table.qza \
  --o-representative-sequences rep-seqs.qza \
  --p-n-threads 0
#=============FEATURE TABLE SUMMARIZE================
qiime feature-table summarize \
  --i-table table.qza \
  --o-visualization table.qzv \
  --m-sample-metadata-file sample-metadata.tsv

qiime feature-table tabulate-seqs \
  --i-data rep-seqs.qza \
  --o-visualization rep-seqs.qzv\
```

3_analysis.sh

```
#BSUB -J whole_jochum_qiime2         #Set the job name to "ExampleJob3"
#BSUB -L /bin/bash                 #Uses the bash login shell to initialize
the job's execution environment.
#BSUB -W 2:00                      #Set the wall clock limit to 24hr
#BSUB -n 40                        #Request 40 cores
#BSUB -R "span[ptile=20]"          #Request 20 cores per node.
#BSUB -R "rusage[mem=2560]"        #Request 2560MB per process (CPU) for the
job
#BSUB -M 2560                      #Set the per process enforceable memory
limit to 2560MB.
#BSUB -o whole_qiimeout.%J              #Send stdout and stderr to
"Example3Out.[jobID]"
#BSUB -e whole_qiimerr.%J

#First Executable Line
module load Anaconda/3-5.0.0.1
source activate qiime2-2018.2
module load R_tamu/3.4.2-intel-2017A-Python-2.7.12-default-mt

qiime alignment mafft \
  --i-sequences rep-seqs.qza \
  --o-alignment aligned-rep-seqs.qza

qiime alignment mask \
  --i-alignment aligned-rep-seqs.qza \
  --o-masked-alignment masked-aligned-rep-seqs.qza

qiime phylogeny fasttree \
  --i-alignment masked-aligned-rep-seqs.qza \
  --o-tree unrooted-tree.qza

qiime phylogeny midpoint-root \
  --i-tree unrooted-tree.qza \
  --o-rooted-tree rooted-tree.qza

qiime diversity core-metrics-phylogenetic --i-phylogeny rooted-tree.qza --i-
table table.qza --p-sampling-depth 1109 --m-metadata-file sample-metadata.tsv
--output-dir ./core-metrics-results

qiime diversity alpha-group-significance --i-alpha-diversity ./core-metrics-
results/faith_pd_vector.qza --m-metadata-file sample-metadata.tsv --o-
visualization ./core-metrics-results/faith-pd-group-significance.qzv

qiime diversity alpha-group-significance --i-alpha-diversity ./core-metrics-
results/faith_pd_vector.qza --m-metadata-file sample-metadata.tsv --o-
visualization ./core-metrics-results/evenness-group-significance.qzv

qiime diversity beta-group-significance --i-distance-matrix ./core-metrics-
results/unweighted_unifrac_distance_matrix.qza --m-metadata-file sample-
metadata.tsv --m-metadata-column Subject --o-visualization ./core-metrics-
results/unweighted-unifrac-round-significance.qzv --p-pairwise

qiime emperor plot \
```

```
  --i-pcoa core-metrics-results/unweighted_unifrac_pcoa_results.qza \
  --m-metadata-file sample-metadata.tsv \
  --p-custom-axes Round \
  --o-visualization ./core-metrics-results/unweighted-unifrac-emperor-
Round.qzv

qiime emperor plot \
  --i-pcoa core-metrics-results/bray_curtis_pcoa_results.qza \
  --m-metadata-file sample-metadata.tsv \
  --p-custom-axes DaysSinceExperimentStart \
  --o-visualization ./core-metrics-results/bray-curtis-emperor-
DaysSinceExperimentStart.qzv
qiime diversity alpha-rarefaction \
  --i-table table.qza \
  --i-phylogeny rooted-tree.qza \
  --p-max-depth 4000 \
  --m-metadata-file sample-metadata.tsv \
  --o-visualization ./alpha-rarefaction.qzv
```

4_workflow.sh

```
#BSUB -J whole_jochum_qiime2         #Set the job name to "ExampleJob3"
#BSUB -L /bin/bash                 #Uses the bash login shell to initialize
the job's execution environment.
#BSUB -W 24:00                      #Set the wall clock limit to 24hr
#BSUB -n 20                        #Request 40 cores
#BSUB -R "span[ptile=20]"          #Request 20 cores per node.
#BSUB -R "rusage[mem=2560]"        #Request 2560MB per process (CPU) for the
job
#BSUB -M 2560                      #Set the per process enforceable memory
limit to 2560MB.
#BSUB -o  whole_qiimeout.%J
#BSUB -e  whole_qiimerr.%J
#First Executable Line
module load Anaconda/3-5.0.0.1
source activate qiime2-2018.2
module load R_tamu/3.4.2-intel-2017A-Python-2.7.12-default-mt

#=========== GREENGENES FEATURE CLASSIFIER ===============
qiime feature-classifier classify-sklearn \
--i-classifier gg-13-8-99-515-806-nb-classifier.qza \
--i-reads  rep-seqs.qza \
--o-classification  gg-13-8-99-taxonomy.qza \
--p-reads-per-batch 0 \
--p-n-jobs -1 \
--p-pre-dispatch 2*n_jobs \
--p-confidence 0.7  \
--verbose \

#===============GREENGENES METADATA TABULATE ======================
qiime metadata tabulate \
--m-input-file gg-13-8-99-taxonomy.qza \
--o-visualization gg-13-8-99-taxonomy.qzv \
qiime tools export gg-13-8-99-taxonomy.qzv \
--output-dir ./greengenes \
#============= GREENGENES TAXA BAR PLOT ====================
qiime taxa barplot \
  --i-table table.qza \
  --i-taxonomy gg-13-8-99-taxonomy.qza \
  --m-metadata-file sample-metadata.tsv \
  --o-visualization gg-13-8-99-tax-bar-plots.qzv

#==============GREENGENES TAXA_COLLAPSE================================
for number in 1 2 3 4 5 6 7
do
    qiime taxa collapse \
       --i-table table.qza \
       --i-taxonomy gg-13-8-99-taxonomy.qza \
        --p-level $number \
        --output-dir './greengenes/'$number

    qiime tools export './greengenes/'$number'/collapsed_table.qza' \
        --output-dir './greengenes/'$number \
done
```

5_closed_ref_for_picrust.sh

```
#BSUB -J whole_jochum_qiime2         #Set the job name to "ExampleJob3"
#BSUB -L /bin/bash                 #Uses the bash login shell to initialize
the job's execution environment.
#BSUB -W 24:00                      #Set the wall clock limit to 24hr
#BSUB -n 20                        #Request 40 cores
#BSUB -R "span[ptile=20]"          #Request 20 cores per node.
#BSUB -R "rusage[mem=2560]"        #Request 2560MB per process (CPU) for the
job
#BSUB -M 2560                      #Set the per process enforceable memory
limit to 2560MB.
#BSUB -o
/scratch/group/ykjolab/jochumHMMEqiime/HMME_QIIME/artifacts/18June18/whole_qi
imeout.%J
#BSUB -e
/scratch/group/ykjolab/jochumHMMEqiime/HMME_QIIME/artifacts/18June18/whole_qi
imerr.%J

#First Executable Line
module load Anaconda/3-5.0.0.1
source activate qiime2-2018.2
module load R_tamu/3.4.2-intel-2017A-Python-2.7.12-default-mt

#================QIIME TOOLS IMPORT=========================
qiime tools import \
--input-path
/scratch/datasets/greengenes_release/gg_13_5/gg_13_8_otus/rep_set/99_otus.fas
ta \
--output-path
/scratch/group/ykjolab/jochumHMMEqiime/HMME_QIIME/artifacts/18June18/greengen
es/gg_13_5_otu_99.qza \
--type 'FeatureData[Sequence]' \

#============= CLOSED REF FOR PICRUST ======================
qiime vsearch cluster-features-closed-reference \
--i-sequences
/scratch/group/ykjolab/jochumHMMEqiime/HMME_QIIME/artifacts/18June18/rep-
seqs.qza \
--i-table
/scratch/group/ykjolab/jochumHMMEqiime/HMME_QIIME/artifacts/18June18/table.qz
a \
--i-reference-sequences
/scratch/group/ykjolab/jochumHMMEqiime/HMME_QIIME/artifacts/18June18/greengen
es/gg_13_5_otu_99.qza \
--p-perc-identity 0.97 \
--output-dir
/scratch/group/ykjolab/jochumHMMEqiime/HMME_QIIME/artifacts/18June18/greengen
es/closedRef_forPIcrust99
```

PICRUSt.sh

```
#==============PICRUST=============================================
#use the feature-table.biom that you exported from the closedrefforPicrust
table
biom-convert feature-table.biom --o closedrefforpicrust.txt --to-tsv \

normalize_by_copy_number.py -i feature-table.biom -o normalized_otus.biom \

predict_metagenomes.py -i normalized_otus.biom -o metagenome_predictions.biom
-a nsti_per_sample.tab --with_confidence \

for number in 1 2 3
do
    categorize_by_function.py -f -i metagenome_predictions.biom -c
KEGG_Pathways -l $number -o predicted_metagenomes.L$number.biom
    biom convert -i predicted_metagenomes.L$number.biom -o
predicted_metagenomes.L$number.txt --to-tsv
done

metagenome_contributions.py -i normalized_otus.biom -o
ko_metagenome_contributions.tab
```

hmme.org

```
sample-id,absolute-filepath,direction
# Lines starting with '#' are ignored and can be used to create
# "comments" or even "comment out" entries
R0h,/scratch/group/ykjolab/jochumHMMMEqiime/trimmed_reads/R0_1_paired.fastq,f
orward
R0h,/scratch/group/ykjolab/jochumHMMMEqiime/trimmed_reads/R0_2_paired.fastq,r
everse
R0c,/scratch/group/ykjolab/jochumHMMMEqiime/trimmed_reads/R0c_1_paired.fastq,
forward
R0c,/scratch/group/ykjolab/jochumHMMMEqiime/trimmed_reads/R0c_2_paired.fastq,
reverse
R0d,/scratch/group/ykjolab/jochumHMMMEqiime/trimmed_reads/R0d_1_paired.fastq,
forward
R0d,/scratch/group/ykjolab/jochumHMMMEqiime/trimmed_reads/R0d_2_paired.fastq,
reverse
R0g,/scratch/group/ykjolab/jochumHMMMEqiime/trimmed_reads/R0g_1_paired.fastq,
forward
R0g,/scratch/group/ykjolab/jochumHMMMEqiime/trimmed_reads/R0g_2_paired.fastq,
reverse
R3h,/scratch/group/ykjolab/jochumHMMMEqiime/trimmed_reads/R3_1_paired.fastq,f
orward
R3h,/scratch/group/ykjolab/jochumHMMMEqiime/trimmed_reads/R3_2_paired.fastq,r
everse
R3a,/scratch/group/ykjolab/jochumHMMMEqiime/trimmed_reads/R3a_1_paired.fastq,
forward
R3a,/scratch/group/ykjolab/jochumHMMMEqiime/trimmed_reads/R3a_2_paired.fastq,
reverse
R3d,/scratch/group/ykjolab/jochumHMMMEqiime/trimmed_reads/R3d_1_paired.fastq,
forward
R3d,/scratch/group/ykjolab/jochumHMMMEqiime/trimmed_reads/R3d_2_paired.fastq,
reverse
R3e,/scratch/group/ykjolab/jochumHMMMEqiime/trimmed_reads/R3e_1_paired.fastq,
forward
R3e,/scratch/group/ykjolab/jochumHMMMEqiime/trimmed_reads/R3e_2_paired.fastq,
reverse
R6h,/scratch/group/ykjolab/jochumHMMMEqiime/trimmed_reads/R6_1_paired.fastq,f
orward
R6h,/scratch/group/ykjolab/jochumHMMMEqiime/trimmed_reads/R6_2_paired.fastq,r
everse
R6a,/scratch/group/ykjolab/jochumHMMMEqiime/trimmed_reads/R6a_1_paired.fastq,
forward
R6a,/scratch/group/ykjolab/jochumHMMMEqiime/trimmed_reads/R6a_2_paired.fastq,
reverse
R6c,/scratch/group/ykjolab/jochumHMMMEqiime/trimmed_reads/R6c_1_paired.fastq,
forward
R6c,/scratch/group/ykjolab/jochumHMMMEqiime/trimmed_reads/R6c_2_paired.fastq,
reverse
R6d,/scratch/group/ykjolab/jochumHMMMEqiime/trimmed_reads/R6d_1_paired.fastq,
forward
R6d,/scratch/group/ykjolab/jochumHMMMEqiime/trimmed_reads/R6d_2_paired.fastq,
reverse
```

APPENDIX B

SAS STATISTICAL ANALYSIS SCRIPTS

PGPR_data_wheat.sas

```
data one;
      title 'PGPR comparison';
      input tmt$ RLENGTH PROJAREA SURFAREA AVGDIAM LPERV ROOTVOL TIP FORK CROSSING SHOOTL SHOOTWETW ROOTWETW
SHOOTDRYW ROOTDRYW SRL SRA BIOMASS;
#DATA SECTION OMITED FROM APPENDIX B
;

proc glm;
      class tmt;
      model RLENGTH PROJAREA SURFAREA AVGDIAM LPERV ROOTVOL TIP FORK CROSSING SHOOTL SHOOTWETW ROOTWETW
SHOOTDRYW ROOTDRYW SRL SRA BIOMASS = tmt;
      means tmt / lsd lines ;

Proc summary nway;
      class tmt;
      var RLENGTH PROJAREA SURFAREA AVGDIAM LPERV ROOTVOL TIP FORK CROSSING SHOOTL SHOOTWETW ROOTWETW SHOOTDRYW
ROOTDRYW SRL SRA BIOMASS;
      output mean=;

Proc print;
run; quit;
```

PGPR_data_maize.sas

```
data one;
title 'PGPR comparison';
input tmt$ RLENGTH PROJAREA SURFAREA AVGDIAM LenPerVol ROOTVOL TIP FORK CROSSING;
datalines;
#DATA SECTION OMITED FROM APPENDIX B;

proc glm;
class tmt;
model RLENGTH PROJAREA SURFAREA AVGDIAM LenPerVol ROOTVOL TIP FORK CROSSING = tmt;
means tmt / lsd lines ;

Proc summary nway;
class tmt;
var RLENGTH PROJAREA SURFAREA AVGDIAM LenPerVol ROOTVOL TIP FORK CROSSING;
output mean=;

Proc print;
run; quit;
```

PGPR_ANOVA.sas

```
data one;
      title 'PGPR comparison';
      input HOST$ REP TMT$ RLENGTH PROJAREA SURFAREA AVGDIAM LenPerVol ROOTVOL TIP FORK CROSSING;
      datalines;
;
#DATA SECTION OMITED FROM APPENDIX B
proc glm;
      class HOST TMT;
      model RLENGTH PROJAREA SURFAREA AVGDIAM LenPerVol ROOTVOL TIP FORK CROSSING = HOST TMT TMT*HOST;


Proc summary nway;
      class HOST TMT;
      var RLENGTH PROJAREA SURFAREA AVGDIAM LenPerVol ROOTVOL TIP FORK CROSSING;
      output mean=;

Proc print;
run; quit;
```

LCMS_pellet.sas

```
data one;
      title 'PGPR LCMS comparison';
      input tmt$ SA BA AZA TenTHOM twelveTHOM IAA;
      datalines;
tmt SA BA AZA TenTHOM twelveTHOM IAA
#DATA SECTION OMITED FROM APPENDIX B
proc glm;
      class tmt;
      model SA BA AZA TenTHOM twelveTHOM IAA = tmt;
      means tmt / lsd lines ;

Proc summary nway;
      class tmt;
      var SA BA AZA TenTHOM twelveTHOM IAA;
      output mean=;

Proc print;
run; quit;
```

LCMS_filtrate.sas

```
data one;
      title 'PGPR LCMS comparison';
      input tmt$ SA BA AZA TenTHOM twelveTHOM IAA;
      datalines;
tmt SA BA AZA TenTHOM twelveTHOM IAA
#DATA SECTION OMITED FROM APPENDIX B
proc glm;
      class tmt;
      model SA BA AZA TenTHOM twelveTHOM IAA = tmt;
      means tmt / lsd lines ;

Proc summary nway;
      class tmt;
      var SA BA AZA TenTHOM twelveTHOM IAA;
      output mean=;

Proc print;
run; quit;
```

HMME_Analysis.sas

```
data one;
      title 'HMME comparison';
      input tmt$ REP RL SFW RFW BIOMASS RTW RDW RWC SURFAREA TIPS R2S S2R SRL SRA;
      datalines;
tmt$ REP RL SFW RFW BIOMASS RTW RDW RWC SURFAREA TIPS R2S
#DATA SECTION OMITED FROM APPENDIX B;

proc glm;
      class tmt;
      model REP RL SFW RFW BIOMASS RTW RDW RWC SURFAREA TIPS R2S S2R SRL SRA=tmt;
      means tmt / lsd lines ;

Proc summary nway;
      class tmt;
      var REP RL SFW RFW BIOMASS RTW RDW RWC SURFAREA TIPS R2S S2R SRL SRA;
      output mean=;

Proc print;
run; quit;
```